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PRINCIPAL INVESTIGATOR: Tony Giordano, Ph.D.

CONTRACTING ORGANIZATION: Louisiana State University Health Sciences Center
Shreveport, LA 71130

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14. ABSTRACT
A number of groups have developed libraries of siRNAs to identify genes through functional genomics. While these studies have validated the approach of making functional RNAi libraries to understand fundamental cellular mechanisms, they require information and knowledge of existing sequences since the RNAi sequences are generated synthetically. An alternative strategy would be to create an RNAi library from cDNA. Unfortunately, the complexity of such a library of siRNAs would make screening difficult. To reduce the complexity, longer dsRNAs could be used; however, concerns of induction of the interferon response and off-target effects of long dsRNAs have prevented their use. As a first step in creating such libraries, long dsRNA was expressed in mammalian cells. The 250-nt dsRNAs were capable of efficiently silencing a luciferase reporter gene that was stably transfected in MDA-MB-231 cells without inducing the interferon response or off-target effects any more than reported for siRNAs. In addition, a long dsRNA expressed in the same cell line was capable of silencing endogenous c-met expression and inhibited cell migration, whereas the dsRNA against luciferase had no effect on c-met or cell migration. The studies suggest that large dsRNA libraries are feasible and that functional selection of genes will be possible.

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RNAi, dsRNA, c-met, cell migration, cancer metastasis

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Introduction

The focus of this Concept Award was to develop methods of generating large dsRNA libraries to silence gene expression in cells. If large dsRNA can be used to effectively silence gene expression, a library could be generated from a cancer cell possessing a phenotype of interest, in this case metastasis, and the library transfected back into the cancer cells. Essential genes that are silenced would be toxic and the cells would be lost; genes silenced that are not involved in metastasis would not prevent cell migration; only those involved in invasion would prevent cell migration and be viable. The plasmids from these non-migrating cells would be isolated and the dsRNA rescued and the sequence determined. If this strategy is to work, long dsRNA is required since the use of siRNA would require a library 10 times as large. However, large dsRNA has been reported to cause the induction of the interferon response leading to cell death and is thought that it would result in significant non-specific, off-target silencing. Thus, the focus of this award was to determine conditions that large dsRNAs could be used to silence gene expression without non-specific or toxic side effects; to show that the silencing of an endogenous gene with a long dsRNA led to functional outcome; generate a library of dsRNAs; and transfect the library and identify novel targets for cell invasion.

Body

There were three main tasks associated with this project. The first was the development of a cell line and functional assay for the RNAi library evaluation, with the creation of the plasmid system the first step in this process. In creating the plasmids we spent a fair amount of time analyzing both convergent polII and polIII promoter systems. The polII system was not successful, likely because of promoter interference, thus we used a polIII system containing U6 and H1 promoters and incorporating T_5 in the at the 3' end of the polylinker for each strand to ensure proper polIII termination of the transcripts. The efficacy of the large dsRNAs generated by this vector and the corresponding off-target and toxicity effects were investigated using the luciferase reporter gene construct. These results were encouraging with good silencing, no induction of the interferon response and off-target effects no worse than those seen with siRNA. This work has been written up and was submitted to *Nucleic Acids Research* last week. A copy of the submitted manuscript is attached as Appendix A. Another aspect of Task 1 was to complete the development of a cre/lox integration system, this has not yet been accomplished. The last aspect of this Task was the work with the matrigel cell invasion assay, which forms the core of Task 2.

The second Task was focused on the generation of dsRNA libraries from highly metastatic cancer cells. Initially, we proposed to use the vector system designed above to silence expression of an endogenous gene, Her2/neu. Since c-met is another endogenous receptor, had been demonstrated to play an important role in cell invasion and is expressed at high levels in the MDA-MB-231 cell line, we decided to focus on its silencing instead of Her2/neu. Results are presented in the above mentioned attached submitted manuscript. To summarize, silencing using this vector system was very efficient and resulted in an inhibition of cell migration, which is extremely encouraging.

While all of the above work suggested that long dsRNA could be used to effectively silence gene expression and not course unwarranted side effects, the project bogged down from here and the remaining Tasks were not able to be completed. The main issue is that highly metastatic tumor cells are NOT truly highly metastatic. That is, for this selection scheme to work, we needed to select a population of cells wherein 99.99% would migrate through a transwell chamber. This population would have been used to generate and transfect the dsRNA library and cells not migrating would be selected. The following scenario can be used to understand the importance of the 99.99% metastatic threshold, using assumptions that favor detection:

Assume transfection efficiency is 100% and that all transfected cells take up only one plasmid;
Assume the frequency of genes involved in metastasis is extremely high, representing 0.5% of the total cDNA;
Assume only 1,000,000 cells are needed to cover the entire library statistically; and
Assume a population of highly metastatic tumor cells is selected wherein 90% migrate.

Thus, 1 million cells would take up a plasmid, of which 5,000 will contain a plasmid involved in cell migration. These 5,000 clones would remain in the top of the transwell chamber representing genes that have been silenced and the cells can no longer migrate. However, 100,000 cells containing plasmids not relevant to cell

migration would also remain in the upper chamber. So the background would be 20-fold higher than the specific silencing.

In this project we started with a highly metastatic MDA-MB-231 cell line and found that 20% of the population actually migrated across the membrane. We used the migrating population, expanded them and placed them back in the transwell chamber but still only 20% migrated. We carried this out a number of times using various chemo attractants but never increased the migrating population to more than 20-30% of the total cell population. In addition, we tried reading cells directly after selection but were also not successful in enriching the population. These studies point out that the majority of highly metastatic cells do not actually migrate and thus the above strategy will not work, the background (noise) would be far too high for selection. Speaking with experts on metastases, this turns out to be an expected finding. They pointed out that even *in vivo* highly metastatic may mean a small percentage of injected tumor cells actually metastasize to other tissues. Of course for human health, any percentage is high, but for the purposes of this selection strategy, the percentage is far to low to be feasible. Thus, will a vector was generated and a long dsRNA to c-met was shown to inhibit cell migration, the major goal of the project, to create libraries of large dsRNAs from highly metastatic cells and use the libraries to silence gene expression and inhibit migration was not accomplished. We have recently submitted an R21 grant to the NCI to try to secure additional funding to test this system using a complicated negative selection strategy. The submitted grant is attached as Appendix B.

Key Research Accomplishments

- Generated dsRNA vector system for production of long dsRNAs
    - Efficient silencing from ¾ test vectors
    - No induction of the interferon cascade
    - Off-target effects somewhat lower than observed with siRNAs
- Silencing of endogenous gene expression (c-met) using long dsRNAs
    - Efficient silencing, comparable to a reported siRNA
    - Inhibition of cell migration

Reported Outcomes

- A vector system for generating long dsRNA (deliverable from Task 1)
- Inhibition of cell migration with c-met long dsRNA
- Manuscript submitted to *Nucleic Acids Research* (Appendix A)
- Poster Presentations
    - 2005 Era of Hope Conference
    - 2005 and 2006 Barlow Symposium at LSUHSC-S
    - 2005 Northern Louisiana Bio Research Day
- No degrees were associated with this work although a PhD student worked on the project and is an author on the submitted manuscript.
- A post-doc worked on this project and will continue similar work at another post-doc position at UNC, Chapel Hill
- Personnel supported by this project
    - Tony Giordano, Ph.D., Project Head and Associate Professor
    - Aurel Strat, MD, Scientist and Post-doc
    - Lu Gao, MD, part of his time, PhD student
- Funding applied for: R21 Innovative Technology Grant from NCI (Appendix B)

Conclusions

The major result from this work was the anticipated yet unexpected finding that long dsRNAs can be used to silence gene expression without inducing the interferon cascade or causing significant off-target effects. This finding will allow investigators to more rapidly target gene expression since the efficiency of silencing is 75% compared to the 25% reported for siRNAs. In addition, using long dsRNAs can have important therapeutic

consequences in the silencing of viruses since resistance is far less likely to develop through escape of silencing. This is because a single mutation in a siRNA can allow the virus to escape silencing whereas a long dsRNA is process into numerous siRNAs intracellularly and thus numerous mutations would be required to allow escape. And for the purposes of this library approach, the ability to use long dsRNAs will provide for a means of generating random libraries cellular RNAs possessing the phenotype of interest, requiring far fewer clones than a similar library constructed using siRNAs for screening, and requiring no prior sequence information of important targets. The failure with this work was the phenotypic selection strategy but a negative selection strategy has now been developed. The ability to generate long dsRNA libraries can also be applied to other phenotypes for which more robust selection strategies are available.

Appendix A

Specific and Nontoxic Silencing in Mammalian Cells with Expressed Long dsRNAs

Aurel Strat, Lu Gao and Tony Giordano*

Department of Biochemistry and Molecular Biology, Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, LA

* Corresponding Author: Tony Giordano
email: agiord@lsuhsc.edu
phone: 318-675-7791
fax: 318-675-5180

Abstract

A number of groups have developed libraries of siRNAs to identify genes through functional genomics. While these studies have validated the approach of making functional RNAi libraries to understand fundamental cellular mechanisms, they require information and knowledge of existing sequences since the RNAi sequences are generated synthetically. An alternative strategy would be to create an RNAi library from cDNA. Unfortunately, the complexity of such a library of siRNAs would make screening difficult. To reduce the complexity, longer dsRNAs could be used; however, concerns of induction of the interferon response and off-target effects of long dsRNAs have prevented their use. As a first step in creating such libraries, long dsRNA was expressed in mammalian cells. The 250-nt dsRNAs were capable of efficiently silencing a luciferase reporter gene that was stably transfected in MDA-MB-231 cells without inducing the interferon response or off-target effects any more than reported for siRNAs. In addition, a long dsRNA expressed in the same cell line was capable of silencing endogenous c-met expression and inhibited cell migration, whereas the dsRNA against luciferase had no effect on c-met or cell migration. The studies suggest that large dsRNA libraries are feasible and that functional selection of genes will be possible.

Introduction

A number of different approaches have been advanced to validate the function of genes, including the function of a number of genes involved in cancer cell migration and invasion. The use of RNAi is generally regarded to be one of the most promising approaches since, at least in non-mammalian systems, it acts systemically (1), thus providing the potential to carry out both *in vitro* and *in vivo* target validation. RNAi was initially described in plants, where it was believed to play a key role in protection against viral pathogens (2). The pathway involves a dsRNA of greater than 21 base pairs triggering an RNaseIII-like enzyme now called Dicer (3-5). Dicer cleaves long dsRNAs, into small interfering RNAs (siRNAs) of 21-25 base pairs. These siRNAs then are incorporated into a multi-subunit RNA-induced silencing complex (RISC), which acts catalytically to target degradation of cellular mRNA in a sequence dependent manner (6, 7). Given the systemic and catalytic nature of RNAi, this class of molecules has been proposed for use both in target identification/validation and the development of therapeutics (8, 9). While the great majority of the target validation studies using RNAi have been focused on a specific target, the efficiency of RNAi has led to the recognition that this technology may represent an exceptionally strong approach to functional genomics through the creation of RNAi libraries.

Libraries of siRNA molecules targeting a specific gene can be produced synthetically, although new enzymatic approaches (10) have reduced the time and costs associated with producing libraries, and allow for the selection of the siRNA with the highest level of activity against the target of interest. Functional RNAi libraries have been designed and synthesized to study cytoskeleton organization in *Drosophila* (11) and are being used extensively to study gene function in *C. elegans,* including the functional analysis of entire chromosomes and the full genome (12, 13) but have only recently been described for use in mammalian systems (14). While the above laboratories reported the ability to generate functional libraries of siRNAs against specific targets and the ability to generate a complex, 415,000 member siRNA library from an existing cDNA library, they did not report that this latter library was functional. The authors go on to report that they generate on average 34 unique siRNA clones per kilobase of sequence, a distinct advantage since not all siRNAs are active. However, this also points out the limitations of generating siRNA libraries. Significantly larger libraries will be required to ensure that active siRNAs are present for each transcript. Other approaches have recently been undertaken to develop functional siRNA libraries ranging from ~500 to 8,000 known genes (15, 16). Plasmids and retroviral vectors have also been used to generate libraries of short hairpin RNAs (shRNAs) of similar size to the larger siRNA library (17, 18), with which genetic screens have been carried out to identify tumor suppressor genes (18-20). Recently, libraries corresponding to most known human and mouse genes have been developed (21). Many of these si-and shRNA libraries are commercially available and a database, RNAi Codex has been developed to annotate the data generated in the various studies (22).

While the above strategies have demonstrated promise for functional genomic screening, most are based on developing libraries using known sequences and when a random library was generated, the complexity was significant. An alternative approach to that taken in generating the random siRNA library (14), would be to restriction digest cDNA to generate a library of long dsRNAs. Long dsRNA libraries have been used to analyze gene function in *Drosophila* (23, 24) but potential off-target effects and induction of the stress response are issues for mammalian gene silencing. Thus, the focus of this study was to analyze the efficiency, selectivity and toxicity of long dsRNAs in mammalian cells and to determine if long dsRNAs lead to the desired biological response in these cells, as a first step to generate long dsRNA libraries.

Materials and Methods

Plasmid construction, primers and probes

For the construction of the vectors containing dual, convergent promoters for expressing dsRNA molecules, two different polIII human promoters, U6 from psiSTRIKE-U6 (Promega) and H1 from pSilencer 3.0-H1 (Ambion), were cloned into pGEM3Z (Promega). The primers used to PCR these promoters (Invitrogen) were designed to contain unique restriction sites at the 5' and 3' ends for cloning and a TTTTT stretch in the reverse primers to create the polIII termination signal:

EcoRI
U6 F: 5'-GCCATG**GAATTC**AGAGGGCCTATTTCCCATG-3'
U6 R: 5'-ATGTAA**GAGCTC**TTTTTCGGTGTTTCGTCCTTTCCAC-3'
SacI

HindIII
H1 F: 5’-GCCGCG**AAGCTT**AATTCATATTTGC-3’
H1 R: 5’- ATCTAA**GGATCC**TTTTTCGAGTGGTCTC-3’
BamHI

The firefly luciferase fragments were generated by PCR from pMS110 (Message Pharmaceuticals) using the following primer pairs (Invitrogen) and cloned into the KpnI site in pGEM/U6/H1:

Forward primer for 250, 500, and 1,000 base pair regions:
5’-ATATAA**GGTACC**ATGGAAGACGCCAAAAAC-3’

Forward primer for 750 base pair region:
5’-ATATAA**GGTACC**ACTCTCTTCAATTCTTTATGCC-3’

Reverse primer for 250 base pair region:
5’-ATATAA**GGTACC**GTTTTCACTGCATACGACG-3’

Reverse primer for 500 base pair region:
5’-ATATAA**GGTACC**GAACGTGTACATCGACTG-3’

Reverse primer for 750 and 1,000 base pair regions:
5’-ATATAA**GGTACC**GCAGATGGAACCTCTTG-3’

The c-met gene (gb accession number: NM_000245) was targeted with a long dsRNA generated by cloning the region between 3209 – 3330 into the KpnI site of pGEM/U6/H1 vector using the following primer pair (Invitrogen):

Forward: 5' - ATATAAGGTACCCAGAAGATCAGTTTCC - 3'
Reverse: 5' - ATATAAGGTACCTGCAGTAATGGACTGG- 3’

The following customized primers and probe sets for qRT-PCR plasmid quantification were purchased from Applied Biosystems:

Primers
Forward (U6): 5’- GCTTACCGTAACTTGAAAGTA- 3’
Reverse (H1): 5’- CTGGGAAATCACCATAAACGT -3’

Luc probe:
5’-CCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCA-3’

c-met probe:
5’- CAGAAGATCAGTTTCCTAATTCATCTCAGAACGGTTCATGCCGACAAGTG-3’

Cell culture and transfection
MDA-MB-231 cells were maintained in DMEM/F12 (1:1), supplemented with 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin, 0.4 mM sodium pyruvate and 2 mM L-glutamine. Cells were transfected with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA) in 96 well, 6 well or T75 flasks as necessary for the desired number of cells.

Luciferase experiments
MDA-MB-231 cells permanently expressing the pMS110 plasmid containing firefly luciferase (MDA-MB-231/FFLuc) were seeded in 96-well culture plates at 10^4 cells/well and transfected with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Each well received 45 ng of the pGEM/U6/H1/dsRNALuc expression vector. All samples were transfected in triplicate and the experiment was performed a minimum of three times. For control transfections, empty vector pGEM/U6/H1 was included. The luciferase assays were performed 24 h after transfection using the Bright Glo Luciferase Assay system according to the manufacturer’s instructions (Promega, Madison, WI).

Transwell migration assay

MDA-MB-231/FFLuc cells were grown in T75 flasks and transfected with the desired dsRNA expression vector. Twenty-four hours following transfection, cells were harvested for the migration assay by trypsinization, suspended in DMEM/F12 serum-free and seeded at densities of 2 x 10^4 or 10^6 cells per well in 24 or 6 well transwell inserts, respectively. The lower chamber was supplemented with DMEM/F12 medium with 10% serum. The upper and lower chambers were separated by a 8 µm pore polycarbonate membrane (Costar, Corning, NY) that was coated with 50µL or 500µL of 0.5µg/µL Matrigel (Becton Dickinson). After 48 h of incubation at 37 °C, the cells in the bottom well were harvested with trypsin and counted for invasion index evaluation (24 well) and RNA or DNA were extraction (6 well) for qRT-PCR and microarray hybridization or plasmid partition quantification, respectively. The number of migrating cells was determined by counting a 10-µl aliquot using a haemocytometer (Neubauer). For each experiment of invasion index evaluation (invasion index = % of cells that migrated from the total number of plated cells), values were normalized to the empty vector transfected cells set at 100%.

RNA isolation and quantitative real-time PCR

Total RNA was extracted with Trizol (Invitrogen) from MDA-MB-231 FFLuc cells 48 hours after transfection with either pGEM/U6/H1 or pGEM/U6/H1dsRNAFFLuc. The estimated transcript concentrations of the human c-met, GAPDH and firefly luciferase genes were based on the comparison of the target transcript PCR signal in dsRNA expression vector transfected cells to the signal measured in an empty vector transfected control. Analysis was done using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (25) for target gene transcripts and interpolation from standard curves for absolute quantification of plasmid partitioning. The TaqMan PCR system (Applied Biosystems) with a 7700 ABI Detector (Applied Biosystems) was used. cDNA was synthesized from 1 µg of the total RNA used in the microarray experiments by use of TaqMan Reverse Transcription Reagents (Applied Biosystems). Pre-designed, gene-specific TaqMan probe and primer sets (Applied Biosystems), consisting of a specific fluorogenic probe and a pair of oligonucleotides, were used to run standard qPCRs for human c-met, GAPDH and firefly luciferase genes. We employed 0.1 ng cDNA for all genes and 5µg DNA for plasmid quantification. The reactions were carried out in triplicate in a 50 µl reaction volume and a 96-well plate format.

Microarray hybridization

Total RNA was extracted as described above. Quality of each sample was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotinylated cRNA was synthesized from total RNA (Enzo, Farmingdale, NY). Following processing according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA), the labeled cRNA was hybridized to Affymetrix U133A GeneChips and the resulting expression levels were quantified. Comparative analysis of the two samples was performed with the statistical algorithm of MAS 5.0 software (Affymetrix) by scaling at fixed target intensity of 150, a fold change of 1.5 (approximate signal log ratio of 0.6) and p value less than 0.01. Comparison results expressed as percentage in the study use the fluorescence signal of the targets on the array.

Statistical analysis

With the exception of the microarray data, all data were analyzed using the Sigma Plot 8.02 statistical software package (SPSS Inc. 2002). All quantitative values were expressed and plotted as mean $\pm$ SEM. Comparisons were evaluated by the unpaired Student's t-test. A value of $p < 0.01$ was considered statistically significant.

Results

To create vectors containing dual, convergent promoters for expressing dsRNA molecules two different polIII promoters, U6 and H1, were cloned into pGEM3Z. The primers used to amplify these promoters were designed to contain unique restriction sites at the 5' and 3' ends for cloning and a TTTTT stretch in the reverse primers to create a strong polIII termination signal. To test various long dsRNAs, regions of the firefly luciferase gene were amplified by PCR and cloned into a *Kpn*I site between the two promoters. Sequences of 250, 500, 750 and 1,000 base pairs were tested for their ability to inhibit firefly luciferase activity in a MDA-MB-231 cell line that stably expressed firefly luciferase. Each of the constructs were capable of silencing luciferase activity 48 hours after transfection with inhibition ranging from 20% for the 500 base pair sequence to 80% for the 250 base pair sequence (Figure 1).

To analyze whether expression of long dsRNAs had adverse effects on the cell, the stress response and off-target effects were monitored. The four constructs were analyzed to determine if expression of long dsRNAs led to the induction of interferon βin order to assess their capability to induce the stress response. The MDA-MB-231 cells, which had previously shown to possess a functional interferon pathway (26), were demonstrated in this study to be β-interferon competent by showing that the cells are permissive and could survive for at least 72 hours following infection with an oncolytic GFP expressing Sindbis virus strain. At 48 hours post transfection, none of the four constructs led to detection of interferon β(Figure 2). Since, the 250 base pair dsRNA resulted in maximal silencing of luciferase without inducing interferon β, this construct was used to analyze off-target effects by expression profiling. Of 22,283 probe sets on the array, 68 were increased by 1.5-fold and 189 decreased by the same level 48 hours after transfection of this construct (Supplemental Data). Interferon β, 2'-5' OAS and PKR were found absent, present, and present with no change in the expression level when compared to vector alone transfected cells, respectively.

While the above results demonstrate that long dsRNA can effectively inhibit the expression of a transgene in MDA-MB-231 cells without inducing the interferon response or strong off-target effects, the ability to silence an endogenous gene and generate the expected phenotypic change with long dsRNA must be assessed. To this end, the ability to silence c-met and prevent cell migration was analyzed. A dsRNA corresponding to the region 3209-3300 of the c-met sequence was cloned into the vector described above and transfected into MDA-MB-231 cells. The 250-base pair luciferase silencing construct was independently transfected into this cell line and 48 hours after transfection, c-met RNA levels were determined using quantitative RT-PCR. The c-met dsRNA construct led to a 60% decrease in c-met RNA levels, whereas the luciferase dsRNA construct did not alter c-met RNA levels (Figure 3).

The transiently transfected cells were then tested for their ability to inhibit cell migration using a transwell assay. The ability of expressed long c-met dsRNA to inhibit migration was compared to that of an siRNA that had been previously demonstrated to efficiently knock down c-met (27). As can be seen in Figure 4, both the expressed long dsRNA and the siRNA inhibited cell migration by ~40%. The inability to inhibit cell migration to higher levels is likely due to the inefficiency of transfection, such that the cells that still migrate may lack plasmid or have significantly less plasmid expressing the dsRNA. To address this prossibility, the partitioning of the plasmids expressing long dsRNA between the migrated and non-migrated cells was quantified using real time PCR to determine the abundance of plasmid DNA per unit of total DNA. Standard curves were generated using the plasmids encoding c-met and luciferase dsRNAs to quantify actual plasmid levels from migrating and non-migrating cells. As expected, the luciferase plasmid was found at relatively similar levels in both the migrating and non-migrating cell populations, whereas the c-met plasmid strongly partitioned with the non-migrating population (Figure 5).

In total, these experiments present data that demonstrate (1) the ability to produce long dsRNA and efficiently silence gene expression when expressed from a plasmid; (2) the lack of interferon induction following transfection and efficient silencing; (3) the ability of a long dsRNA (c-met) to inhibit migration; and, (4) the increased partitioning of this plasmid (c-met) with the non-migrating cells since silencing of c-met would be expected to lead to prevention of migration. Similar expression of long dsRNA targeting Arc and NR1 in a neuronal cell line have also been carried out and confirm the above observations (data not shown).

Discussion

The above studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects. One concern with using long dsRNAs randomly generated from cDNA libraries and expressed from promoters constructed of two convergent polIII promoters is premature termination of the transcript due to the presence of polIII terminators. A long stretch of thymidine nucleotides leads to polIII termination, with a minimum of three necessary but five being optimal (28). Thus, if a terminator was present in either the sense or antisense strand very near to the promoter or if terminators for both strands were present adjacent to one another, it would be possible to generate no dsRNA. It was interesting to note that the optimal long dsRNA for silencing luciferase expression was in fact, the shortest of the sequences. However, if the sequence was analyzed for stretches of five thymidine nucleotides in either the forward or reverse orientation, the 250 base pair sequence would generate a region of ~235 base pairs of dsRNA, whereas the 750 and 1,000 base pair sequences would generate identical ~330 base pairs of dsRNA and the 500 base pair sequence would result in a dsRNA of ~80 base pairs, perhaps explaining its poor

silencing activity. These results demostrate that while the existance of polIII terminators may prevent some dsRNAs from forming, a stretch of five thymidines should occur on average once every 1,000 base pairs so the likelihood that such stretches will occur on opposite strands of a 250 nucleotide base pair digestion product is remote. In addition, it is extremely unlikely that if a cDNA was digested to numerous 250 base pair fragments, every fragment would contain terminators preventing formation of dsRNAs. Thus, use of random 250 base pair regions should result in effective silencing.

Long dsRNAs are capable of undergoing dicer-mediated processing to siRNAs in mammalian cells resulting in the production of numerous siRNAs of 22-23 nucleotides (29). The presence of numerous fragments against a single target has the advantage of allowing the cell to select the most efficient siRNA for target accessibility and by producing numerous dsRNAs, can offer better potential therapeutic protection against viral infections by making resistance difficult (9). However, there has been a long standing belief that long dsRNAs induce an interferon response, beginning with dsRNA viruses induction of a stress response (30-32) and further supported when early work comparing long dsRNA to siRNAs was carried out (7). These studies are often cited as rationale for working with siRNAs but other studies have reported that siRNA can also induce an interferon response in mammalian cells (33, 34). Although siRNAs are still the method of choice for silencing gene expression, there have been a number of reports in which long dsRNAs have been used in mammalian cells and in mammals without signs of toxicity or adverse effects on the animal (35-38). The lack of interferon response associated with long dsRNA in this study was an encouraging first step in the development of libraries since long dsRNA would reduce the required complexity and allow processing to numerous siRNAs intracellularly enhancing the probability of target acessibility.

A second important consideration when using long dsRNA is the potential for significant off-target silencing. One of the proposed strengths of an siRNA is that the 21-23 base pair sequence can be analyzed against the genomic database and identical matches can be avoided. This becomes difficult, if not impossible, when using a 250 base pair dsRNA that will be processed into a number of potentially overlapping 21-23 base pair siRNAs (29). One of the real strengths of siRNAs was originally thought to be the exquisite specificity of the interaction. When a reporter gene, GFP, was used as a target to assess silencing ot the reporter expression in HEK293 cells, no detectable secondary effects were noted by genomewide expression profiling (39) with a similar profiling demonstrating high specificity when the profiles of three endogenous genes were compared (40). The tremendous specificity of the response was also inferred from data generated using siRNAs as antiviral treatments – resistant viruses were shown to contain a *single* nucleotide substitution in the region corresponding to the sequence of the siRNA (41). More recently, this specificity has come into question. When a similar expression profiling was carried out using multiple siRNAs against a MAPK14 or IGF1R, there were reproducible expression patterns for each siRNA across three experiments but a set of siRNAs against a given target found only a few genes regulated similarly for the given target (42). This was unexpected since siRNAs targeting the same gene should result in the same changes in an expression profile. They also reported that the observed off-target effects could not be titrated out using lower concentrations of siRNA and that an siRNA targeting luciferase reproducibly silenced other genes in the absence of any sequence similarity. An 21 base pair siRNA against luciferase was also found to elicit significant off-target effects, stimulating or repressing >1,000 genes (43). They explained this, at least in part, through the induction of the stress response through the PKR pathway. In light of these latter studies, the several hundred genes identified by expression analysis in this study that exhibit an increase or decrease following silencing of luciferase with long dsRNA were not unexpected for silencing in general. From these results, long dsRNAs do not appear to be toxic and the non-specific effects are modest when compared to those of siRNAs.

We next tested whether a long dsRNA expressed from a plasmid could not only silence gene expression but also to result in a desired phenotypic change. To analyze this activity we choose to attempt to inhibit cell migration as a strategy for targeting cancer metastasis. The understanding of how a primary tumor cell migrates from the initial tumor, disperses throughout the body in the vascular/lymphatic system, invades a new site and begins to proliferate and form secondary tumors in not well understood (44), although it is well accepted that the mechanisms leading to metastases will represent attractive therapeutic targets (45). Since migration and invasion are key components of cellular metastasis (45, 46), these processes have been well studied in cell culture systems and represent an attractive model to look at the functional effects of large dsRNAs.

Recently, an siRNA to c-met has been identified and shown to lead to apoptosis and reduction of tumor growth (27). In addition, when c-met was knocked-down using ribozymes, cell invasion and metastasis were inhibited both in vitro and in mice (47, 48). A number of groups are targeting c-met for therapeutic intervention with small molecules (49, 50) given its important role in tumor metastasis (51). For these reasons, c-met was used in the present studies as a positive control dsRNA for cell invasion. When comparing the dsRNA to the previously reported siRNA, both inhibited cell migration to an identical level. To determine whether this inhibition was due to the transfection of the plasmid containing the dsRNA for c-met, the migrating and non-migrating populations of cells were compared. While a plasmid expressing dsRNA against luciferase was found in equal abundance in the two populations of cells, the plasmid expressing dsRNA against c-met was enriched almost four-fold in the non-migrating cell population. This enrichment is consistent with the loss of c-met function, which should result in inhibition of migration. Thus, these studies demonstrate the ability to silence an endogenous gene and obtain the desired phenotype. The presence of c-met dsRNA plasmid in the migrating population of cells may represent low number of copies per cell resulting in low levels of dsRNA that are insufficient to elicit the change in migration or may represent a population of the MDA-MD-231 cells whose migration is not dependent on c-met expression. A further analysis of this population would be required to better understand the biological significance of this phenomenon.

Results from these studies have demonstrated the ability of dsRNA to silence endogenous gene expression, leading to a biological response, without inducing an interferon response or significant off-target effects. This is the first step in creating functional dsRNA libraries. By using long dsRNAs, libraries can be generated from cells expressing a phenotype of interest and transfected back into the same type of cells to look for loss of function. The dsRNA sequence resulting in loss of function can then be rescued, sequenced and the isolated sequence further tested for function. The use of long dsRNAs also offer promise for development of therapeutics, particularly since off-target effects are modest when compared to those found with siRNAs.

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References

1. Winston, W. M., Molodowitch, C., and Hunter, C. P. Systemic RNAi in C. elegans Requires the Putative Transmembrane Protein SID-1. Science, *295:* 2456-2459, 2002.
2. Baulcombe, D. RNA silencing in plants. *431:* 356-363, 2004.
3. Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev., *13:* 3191-3197, 1999.
4. Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *404:* 293-296, 2000.
5. Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals. Cell, *101:* 25-33, 2000.
6. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. Science, *293:* 1146-1150, 2001.
7. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *411:* 494-498, 2001.
8. Chopra, M., Pachuck, C., Satishchandran, C., Giordano, T. Using RNA interference to modulate gene expression. Targets, *1*, 2002.
9. Shuey, D., McCallus, DE, Giordano, T. RNAi: gene-silencing in therapeutic intervention. Drug Discovery Today, *7:* 1040-1046, 2002.
10. Shirane, D., Sugao, K., Namiki, S., Tanabe, M., Iino, M., and Hirose, K. Enzymatic production of RNAi libraries from cDNAs. *36:* 190-196, 2004.
11. Kiger, A., Baum, B., Jones, S., Jones, M., Coulson, A., Echeverri, C., and Perrimon, N. A functional genomic analysis of cell morphology using RNA interference. Journal of Biology, *2:* 27, 2003.
12. Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. *408:* 325-330, 2000.

13. Sonnichsen, B., Koski, L. B., Walsh, A., Marschall, P., Neumann, B., Brehm, M., Alleaume, A.-M., Artelt, J., Bettencourt, P., Cassin, E., Hewitson, M., Holz, C., Khan, M., Lazik, S., Martin, C., Nitzsche, B., Ruer, M., Stamford, J., Winzi, M., Heinkel, R., Roder, M., Finell, J., Hantsch, H., Jones, S. J. M., Jones, M., Piano, F., Gunsalus, K. C., Oegema, K., Gonczy, P., Coulson, A., Hyman, A. A., and Echeverri, C. J. Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans. *434:* 462-469, 2005.
14. Sen, G., Wehrman, T. S., Myers, J. W., and Blau, H. M. Restriction enzyme-generated siRNA (REGS) vectors and libraries. *36:* 183-189, 2004.
15. Aza-Blanc, P., Cooper, C. L., Wagner, K., Batalov, S., Deveraux, Q. L., and Cooke, M. P. Identification of Modulators of TRAIL-Induced Apoptosis via RNAi-Based Phenotypic Screening. Molecular Cell, *12:* 627-637, 2003.
16. Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S., and Schultz, P. G. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. PNAS, *101:* 135-140, 2004.
17. Paddison, P. J., Silva, J. M., Conklin, D. S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K., Chang, K., Westbrook, T., Cleary, M., Sachidanandam, R., Richard McCombie, W., Elledge, S. J., and Hannon, G. J. A resource for large-scale RNA-interference-based screens in mammals. *428:* 427-431, 2004.
18. Berns, K., Hijmans, E. M., Mullenders, J., Brummelkamp, T. R., Velds, A., Heimerikx, M., Kekhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P.S., Beijersbergen, R.L., Bernards, R. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature, *428:* 431-437, 2004.
19. Kolfschoten, I. G. M., van Leeuwen, B., Berns, K., Mullenders, J., Beijersbergen, R. L., Bernards, R., Voorhoeve, P. M., and Agami, R. A Genetic Screen Identifies PITX1 as a Suppressor of RAS Activity and Tumorigenicity. Cell, *121:* 849-858, 2005.
20. Westbrook, T. F., Martin, E. S., Schlabach, M. R., Leng, Y., Liang, A. C., Feng, B., Zhao, J. J., Roberts, T. M., Mandel, G., and Hannon, G. J. A Genetic Screen for Candidate Tumor Suppressors Identifies REST. Cell, *121:* 837-848, 2005.
21. Silva, J., Li, MZ, Chang, K, Ge, W, Golding, MC, Rickles, RJ, Siolas, D, Hu, G, Paddison, PJ, Schlabach, MR, Sheth, N, Bradshaw, J, Burchard, J, Kulkarni, A, Cavet, G, Sachidanandam, R, McCombie, WR, Cleary, MA, Elledge, SJ, Hannon, GJ. Second-generation shRNA libraries covering the mouse and human genomes. Nature Genetics, *37:* 1281-1288, 2005.
22. Olson, A., Sheth, N., Lee, J. S., Hannon, G., and Sachidanandam, R. RNAi Codex: a portal/database for short-hairpin RNA (shRNA) gene-silencing constructs. Nucl. Acids Res., *34:* D153-157, 2006.
23. Ivanov, A. I., Rovescalli, A. C., Pozzi, P., Yoo, S., Mozer, B., Li, H.-P., Yu, S.-H., Higashida, H., Guo, V., Spencer, M., and Nirenberg, M. Genes required for Drosophila nervous system development identified by RNA interference. PNAS, *101:* 16216-16221, 2004.
24. Kim, Y.-O., Park, S.-J., Balaban, R. S., Nirenberg, M., and Kim, Y. A functional genomic screen for cardiogenic genes using RNA interference in developing Drosophila embryos. PNAS, *101:* 159-164, 2004.
25. Livak, K. J. and Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. Methods, *25:* 402-408, 2001.
26. Vidalain, P.-O., Laine, D., Zaffran, Y., Azocar, O., Servet-Delprat, C., Wild, F., Rabourdin-Combe, C., Valentin, H. Interferons Mediate Terminal Differentiation of Human Cortical Thymic Epithelial Cells. J. Virol., *76:* 6415-6424, 2002.
27. Shinomiya, N., Gao, C. F., Xie, Q., Gustafson, M., Waters, D. J., Zhang, Y.-W., and Vande Woude, G. F. RNA Interference Reveals that Ligand-Independent Met Activity Is Required for Tumor Cell Signaling and Survival. Cancer Res, *64:* 7962-7970, 2004.
28. Bakken, A., Morgan, G., Sollner-Webb, B., Roan, J., Busby, S., and Reeder, R.H. Mapping of transcription initiation and termination signals on Xenopus laevis ribosomal DNA. Proc. Natl. Acad. Sci. U.S.A., *79:* 56-60, 1982.
29. Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. PNAS, *98:* 14428-14433, 2001.
30. Clemens, M., Elia, A. The double-stranded RNA-dependent protein kinase PKR: structure and function. J Interferon Cytokine Res, *17:* 503-524, 1997.
31. Gil, J. s. and Esteban, M. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. Apoptosis, *5:* 107-114, 2000.
32. Manche, L., Green, S. R., Schmedt, C., and Mathews, M. B. Interactions Between Double-Stranded RNA Regulators and the Protein Kinase DAI. Mol Cell Biol, *12:* 5238-5248, 1992.

33. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H., and Williams, B. R. G. Activation of the interferon system by short-interfering RNAs. *5:* 834-839, 2003.
34. Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A.-L., and Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. *34:* 263-264, 2003.
35. Bhargava, A., Dallman, M. F., Pearce, D., and Choi, S. Long double-stranded RNA-mediated RNA interference as a tool to achieve site-specific silencing of hypothalamic neuropeptides. Brain Research Protocols, *13:* 115-125, 2004.
36. Diallo, M., Arenz, C., Schmitz, K., Sandhoff, K., and Schepers, U. Long Endogenous dsRNAs Can Induce Complete Gene Silencing in Mammalian Cells and Primary Cultures. Oligonucleotides, *13:* 381-392, 2003.
37. Paddison, P. J., Caudy, A. A., and Hannon, G. J. Stable suppression of gene expression by RNAi in mammalian cells. PNAS, *99:* 1443-1448, 2002.
38. Tran, N., Raponi, M., Dawes, I. W., and Arndt, G. M. Control of specific gene expression in mammalian cells by co-expression of long complementary RNAs. FEBS Letters, *573:* 127-134, 2004.
39. Chi, J.-T., Chang, H. Y., Wang, N. N., Chang, D. S., Dunphy, N., and Brown, P. O. Genomewide view of gene silencing by small interfering RNAs. PNAS, *100:* 6343-6346, 2003.
40. Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N., and Fesik, S. W. Specificity of short interfering RNA determined through gene expression signatures. PNAS, *100:* 6347-6352, 2003.
41. Gitlin, L., Karelsky, S., and Andino, R. Short interfering RNA confers intracellular antiviral immunity in human cells. *418:* 430-434, 2002.
42. Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. Expression profiling reveals off-target gene regulation by RNAi. *21:* 635-637, 2003.
43. Persengiev, S. P. and Zhu, X., Green, M. R. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA, *10:* 12-18, 2004.
44. Welch, D. R., Steeg, P. S., and Rinker-Schaeffer, C. W. Molecular biology of breast metastasis: Genetic regulation of human breast carcinoma metastasis. Breast Cancer Res, *2:* 408 - 416, 2000.
45. Sledge, G. W. and Miller, K. D. Exploiting the hallmarks of cancer. European Journal of Cancer, *39:* 1668-1675, 2003.
46. Hanahan, D. and Weinberg, R. A. The Hallmarks of Cancer. Cell, *100:* 57-70, 2000.
47. Kim, S. J., Johnson, M., Koterba, K., Herynk, M. H., Uehara, H., and Gallick, G. E. Reduced c-Met Expression by an Adenovirus Expressing a c-Met Ribozyme Inhibits Tumorigenic Growth and Lymph Node Metastases of PC3-LN4 Prostate Tumor Cells in an Orthotopic Nude Mouse Model. Clin Cancer Res, *9:* 5161-5170, 2003.
48. Davies G, W. G., Mason MD, Jiang WG. Targeting the HGF/SF receptor c-met using a hammerhead ribozyme transgene reduces in vitro invasion and migration in prostate cancer cells. Prostate, *60:* 317-324, 2004.
49. Christensen, J. G., Burrows, J., and Salgia, R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Letters, *225:* 1-26, 2005.
50. Wang, X., Le, P., Liang, C., Chan, J., Kiewlich, D., Miller, T., Harris, D., Sun, L., Rice, A., Vasile, S., Blake, R. A., Howlett, A. R., Patel, N., McMahon, G., and Lipson, K. E. Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. Mol Cancer Ther, *2:* 1085-1092, 2003.
51. Corso, S., Comoglio, P. M., and Giordano, S. Cancer therapy: can the challenge be MET? Trends in Molecular Medicine, *11:* 284-292, 2005.

Figure Legends

Figure 1: Firefly luciferase gene silencing in transiently transfected MDA MB231 permanently expressing firefly luciferase cells with long dsRNA expressed from the pGEM/U6/H1 vector. Luciferase activity was analyzed 48 hours following transfection of the vectors expressing dsRNA. Firefly luciferase activity was used as a read out and normalized to vector control.
$p < 0.01$(★).

Figure 2: Interferon beta production at 48 hours after transfection in the supernatant of MDA-MB-231 FFLuc cells transfected with various dsRNA expression vectors, measured by ELISA (OD = 0.046 cut-off corresponds to 0 UI of IFN-beta).

Figure 3: Cells were transiently transfected with vector alone, a vector expressing 250 base pairs of luciferase dsRNA or a vector expressing 121 base pairs of c-met dsRNA using Lipofectamine2000. 48 hours after transfection, total RNA was isolated using Trizol and levels of c-met were quantified. The levels of c-met RNA were expressed relative to those found in vector alone transfected cells. $p < 0.01$(★).

Figure 4: MDA-MB-231 cells were transiently transfected with either a plasmid encoding c-met long dsRNA or c-met siRNA by Lipofectamine 2000. Twenty-four hours after transfection, cells were added to a transwell chamber coated with matrigel, allowed to migrate for 48 hours and the number of cells from the lower compartment of the chamber was compared to those transfected with vector alone. $p < 0.01$(★).

Figure 5: MDA-MB-231 cells were transiently transfected with a plasmid capable of generating either c-met or luciferase long dsRNA. Following a transient transfection, the cells were placed in a transwell chamber coated with matrigel and 48 hours later the migrated and non-migrated populations of cells were isolated. DNA was recovered from each population and the levels of plasmid were analyzed using primers derived from the U6 and H1 promoters by qPCR using the ABI7700. $p < 0.01$(★).

Figures

Figure 1

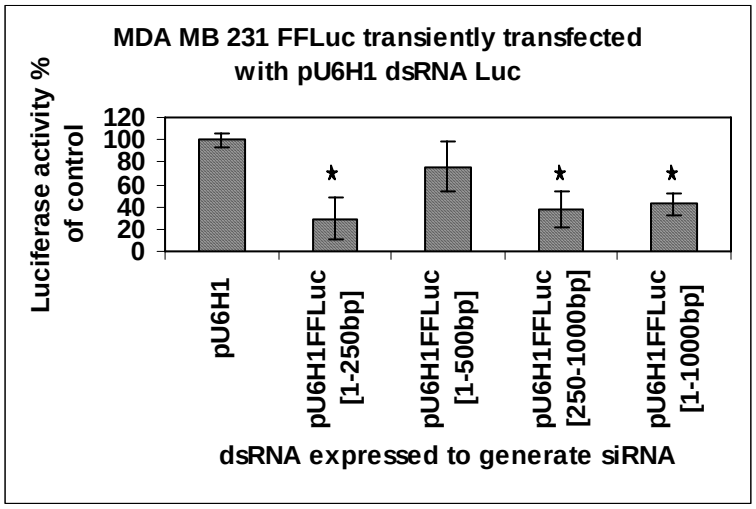
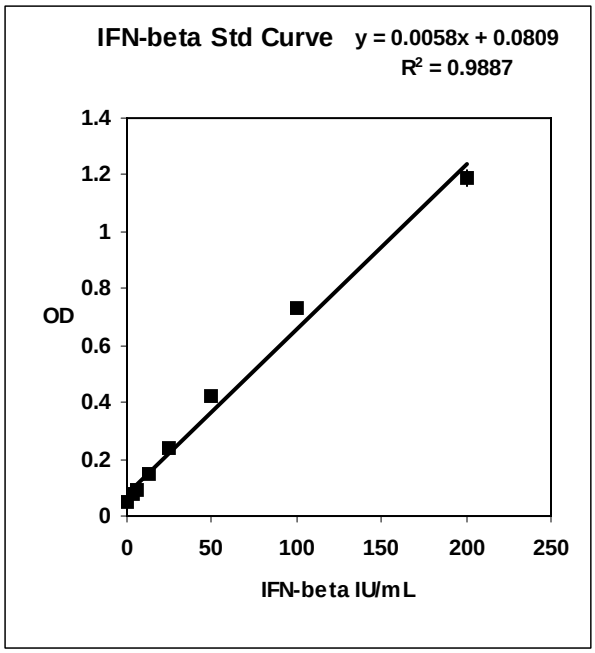


Figure 2


IFN-beta Std Curve
y = 0.0058x + 0.0809
R2 = 0.9887
OD
IFN-beta IU/mL
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
50
100
150
200
250

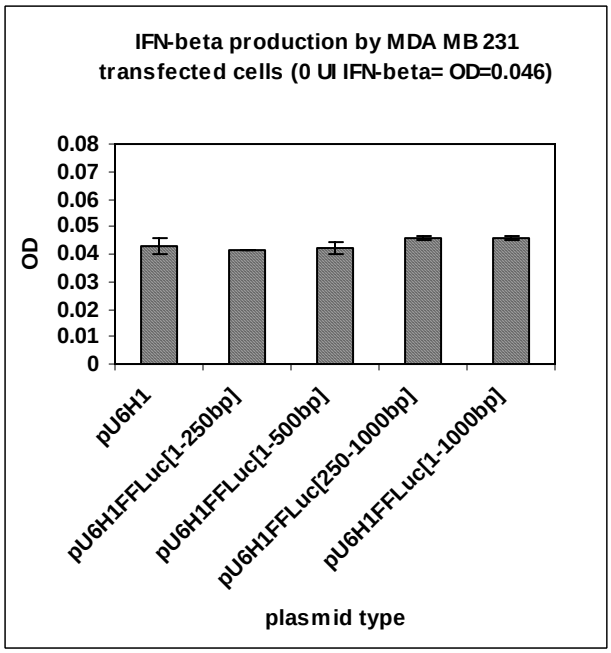

IFN-beta production by MDA MB 231
transfected cells (0 UI IFN-beta= OD=0.046)
OD
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
pU6H1
pU6H1FFLuc[1-250bp]
pU6H1FFLuc[1-500bp]
pU6H1FFLuc[250-1000bp]
pU6H1FFLuc[1-1000bp]
plasmid type

Figure 3

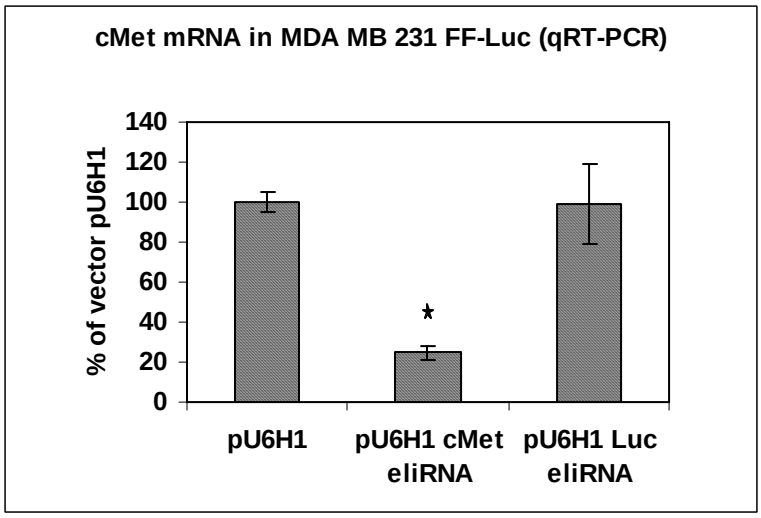

cMet mRNA in MDA MB 231 FF-Luc (qRT-PCR)
% of vector pU6H1
140
120
100
80
60
40
20
0
*
pU6H1
pU6H1 cMet eliRNA
pU6H1 Luc eliRNA

Figure 4

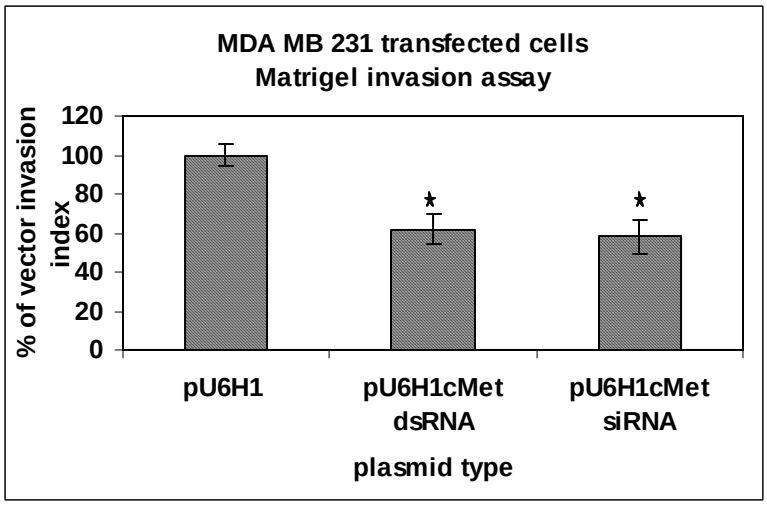
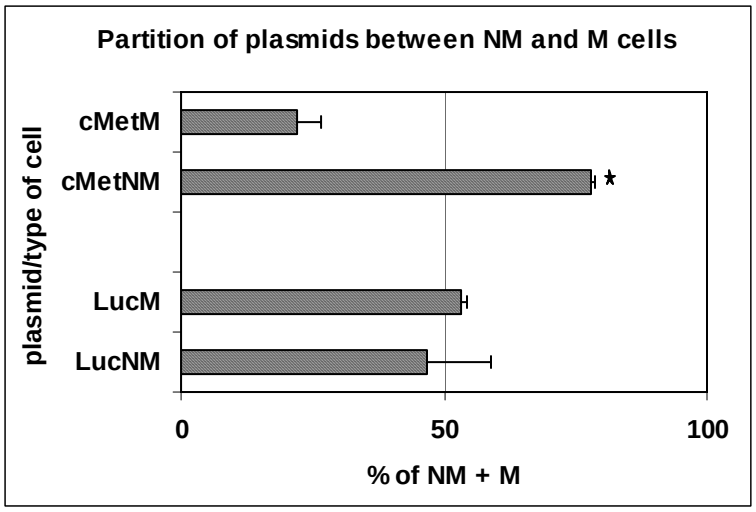

MDA MB 231 transfected cells
Matrigel invasion assay
% of vector invasion index
120
100
80
60
40
20
0
pU6H1
pU6H1cMet dsRNA
pU6H1cMet siRNA
plasmid type

Figure 5


Partition of plasmids between NM and M cells
plasmid/type of cell
cMetM
cMetNM
LucM
LucNM
*
0
50
100
% of NM + M

APPENDIX B

Introduction to Revised Application

This is a second revision of an application originally submitted in February of 2004 that incorporates most of the suggestions made by the reviewers. Again, I appreciate the reviewers' comments that this is an extremely innovative application and the proposed work is of high significance. In both the summary and individual critiques, the reviewers state that previous comments have been effectively addressed. In the review of the revised application, a number of additional concerns were raised. A general feeling is that this is a high risk but innovative application. I do agree with this assessment and feel that makes this appropriate for this RFA that calls for "Innovative Technologies", which would seem by definition to call for high risk types of proposals. I believe that even if the main goal of this application is not achieved, that is the identification of novel genes involved in metastasis, the technology developed along the way will be innovative and could likely play a role in development of additional libraries targeting less complex phenotypes and may be of use in the over-all field of gene silencing since successful implementation of the earlier specific aims will result in the ability to express large dsRNA. For these reasons, while I certainly acknowledge that the biology is high-risk, I believe the technology advancements are within reach.

The first reviewer presents valid concerns around a) the creation of long dsRNA silencing vectors, b) the ability to integrate single copies, c) the unpredictability of the interferon response and d) complexity involved in the loss of migration. The reviewer further recommends that more attention should be given to the use of a positive control. I have now added data to Section C that I hope alleviates some of these concerns. First, in response to a) and c), I have added additional data showing the ability of a vector that expresses long dsRNA, described in the first revised application, to silence gene expression and also the lack of interferon response in cells lines transfected with this vector. We have also used long dsRNA in another project involving the silencing of two different neuronal genes and have been able to select stable cell lines in which each gene is silenced. The ability to derive stable cells lines demonstrates the lack of activation of the interferon pathway even over long periods of time. Since a vector has been developed that allows expression of long dsRNA without activating the interferon response in a number of cell lines, Specific Aim 1b has been replaced with a new Specific Aim 1c, a back-up strategy for functional assay selection of cell invasion. Comment b) is addressed in Specific Aim 1a where I describe using restriction mapping to ensure single integrants in the original cell line. Also, we have now generated data with c-met dsRNA and its use as a positive control in invasion and have incorporated this data in Section C and the use of this construct in the experimental design.

The last concern of the first reviewer and the major concern of the third reviewer is likewise both important and relevant and, in fact, what I feel is the highest risk associated with this proposal. As I mentioned above, I strongly believe that we are and will overcome the technical, molecular hurdles associated with development of this novel phenotypic library selection method. A major question however is the biology of cell migration. The third reviewer correctly points out that highly invasive may not be sufficiently invasive to reduce the background levels and that while this strategy may be successful, only time will tell. Similarly, the first reviewer states that the cell selection strategy may not be sufficiently robust. Since both reviewers are likely experts in the field of migration, these concerns must be taken serious. However, both seem to say that it *might* not work and not that it won't be successful. Thus, in response to both reviewers concerns, I have added the use of c-met dsRNA to the original Specific Aim 1c (now 1b) and discussed the need for potential continued rounds of selection with enrichment of important genes in each round. I have also added an alternative strategy as a new Specific Aim 1c that relies on negative selection. While I believe that these represent novel and innovative strategies for overcoming the complex biology issues associated with cell invasion, I also believe that even if these strategies fail, the libraries produced will have utility for analysis of other cancer phenotypes.

All changes in this second revision are identified by italics. Original revisions in response to the first review have been incorporated into the text. Below is the response (edited to fit page limitations) to the first review for the benefit of any new reviewers:

I appreciate the reviewers comment that if successful, this will be highly significant and hope to clear up some of their concerns, many of them appropriate and important. Two general comments/criticisms of the original proposal are that it was overly ambitious and that the use of expression-based dsRNA (or presumably, any RNAi molecule) faces the same challenges that have limited the effectiveness of antisense as a therapeutic. This revised application seeks to answer both of these criticisms by defining the focus much more on the validation of novel targets as opposed to the therapeutic potential of RNAi molecules. As the reviewers stated, the development of a bulk RNAi based screen to identify new pathways and mechanisms is itself a major achievement and while I had intended for this to be the focus of the original application upon rereading

the application I realized that many of the studies were focused on the therapeutic potential of the molecules. I have removed these studies (Specific Aim 4) since the addition of the synergy studies and other therapeutic work will be better carried out in a subsequent R33. The focus of this application will be to develop the screening technology and validate the approach by isolating novel genes involved in metastasis.

In the summary, the reviewers also state that vector construction is quite complex and may result in self-silencing. We have recently constructed the dual polIII vector and will be testing it shortly but have included references from others who have successfully worked with dual promoters, which should alleviate some of these concerns. I have hopefully addressed the concern over the interferon response by proposing to create a cell line, if necessary, in which interferon expression is silenced. Doxorubicin has been removed as a control for the metastasis assay with the established RNAi molecules used instead, which, I agree with the reviewers, is a much better control. The final comment in the summary actually speaks to the very purpose for carrying out this work. I strongly agree with the reviewers' comment that the failure of invasion inhibitors may not be due to a failure in inhibiting the target but that target inhibition doesn't result in in vivo efficacy. This suggests that the current targets are not appropriate and would argue for the identification of new targets.

In critique 1, the reviewer notes that the 4-6 day silencing using siRNA was not as impressive as that noted in some recent publications, which I agree with. Recent work with novel chemistries by Sirna, Inc. and Alnylam has demonstrated stable silencing for up to 30 days. For therapeutic purposes this is certainly significant and important, however, for the purposes of a bulk RNAi screen, vectors must be used so that the RNAi sequence can be isolated. The fact that silencing exists for at least 10 days using vectored dsRNA should provide sufficient time to carry out the proposed experiments.

This reviewer sites two weaknesses the first, more of a question concerning what has been done and what needs to be carried out. The original vectors used are the property of the company (Nucleonics) and thus must be recreated. Most important, the library work was never attempted as Nucleonics focused its efforts on HBV therapeutics. Thus, while there is preliminary evidence suggesting that this strategy will be successful, the library and phenotypic selection remains to be tested. The second weakness as seen by this reviewer is that the studies with the chemotherapeutic agents may be speculative and overly ambitious. As discussed above, these experiments have been eliminated from this revised application.

In critique 2, the reviewer is very complementary in that this strategy represents a major step forward to be able to do bulk RNAi screening without knowledge of the specific target but seems concerned about the ambitiousness and complexity of the proposal, the choice of target and the delivery issues when contemplating RNAi as a therapeutic, all of which are very legitimate concerns. In response to the concern over the ambitious, labor-intensive and complicated proposal, I have scaled back on the ambitiousness of the proposal by focusing the grant on the screening strategy and not trying to fully characterize the resulting targets. There are still a number of variables, such as optimal promoters, the interferon response and single-integrants that must be addressed but to develop a core screening strategy, I believe that these are fundamental issues and by eliminating the compound studies, more time can be focused on these parameters. As for the choice of the initial target for developing this platform, I think the reviewer's primary concern is lack of drugs and not necessarily the phenotypic assay which will be used to develop the platform. As mentioned above, I would agree that the lack of drugs in this area is noteworthy but feel that this may be in part due to the lack of the appropriate target. At a minimum, the successful completion of this proposal will result in new targets that can be used to screen for drugs and a platform that can be used for isolation of genes involved in other disease related processes. I also appreciate his final comment in the summary section: this is sophisticated molecular biology. As a molecular biologist, I am glad to hear this. Obviously, he goes on to say that the cell biology presented is naïve, which I think is fair. As the head of research at various organizations, I have not focused specifically on any one disease or process. As a member of the Feist-Weiller Cancer Center (FWCC), I am now much more involved in the understanding of cancer biology. I have recently joined the existing Cancer Cell Metastasis Group at the FWCC. Nonetheless, I have only been focused in this area for one year and have thus taken this reviewer's comment seriously and have asked the Head of Research at the FWCC and initiator of the Cancer Cell Metastasis Group, Dr. Jim Cardelli, to serve as an advisor to me on this project. Furthermore, as a member of the Group, I will present my results at least once every quarter to the entire group.

The reviewer asks that the number of stable clones and β-gal expression levels be quantified, which has been added to the revised application. The reviewer is concerned about the use of dual promoters and citations have been added to address this issue, see the "Analyze expression systems (i.e., dual polIII promoters) using luciferase and Her2/neu" section of Specific Aim 1, along with the actual strategies for developing the vectors internally. His/her comments on the complexity of the interferon response are justified and to eliminate this in the present program, interferon itself will be silenced, if necessary, or shorter RNA

sequences will be required. Nevertheless, the response will be studied to determine if we can overcome size constraints in generating subsequent libraries for investigation of other phenotypes. While I agree that this is a complex issue, to develop this as a broad platform, the issue needs to be addressed. I appreciate his encouragement of testing the system with Her2/neu. As mentioned above, I have removed the use of doxorubicin for validating the assay and will instead stick with the siRNAs.

The reviewer's comments about the failure of invasion inhibitors have been addressed above but I will reiterate that I agree with him 100%. The lack of successful inhibitors is the result of either poor targets or a system too complex to inhibit with a single molecule. If the former is true, this project will provide new targets which may overcome this failure, if not by RNAi, then with traditional small molecules. I would agree with the reviewer that eRNAi against existing targets is unlikely to be successful where small molecules have failed and thus the proposal is not to substitute eRNAi for small molecules, rather it is to find new targets amenable to regulation by either small molecules or eRNAi. If the latter is true, and the system is simply to complex to address targeting a single molecule, this work will have still significantly impacted on cancer analysis by providing an innovative strategy for identifying cancer targets and analyzing pathways. This strategy could be used to better understand other mechanisms involved in cancer onset, progression and drug resistance. The reviewer is very negative about the in vitro chemosynergy studies and these will be removed as the therapeutic aspects of this proposal are removed. This may be one place where I showed my naivete but it was my thought that as cancer cells become more metastatic they also become more resistant to cytotoxins which is why metastasis is so disastrous. By preventing metastasis, I rationalized that additional changes in gene expression could also be prevented rendering the cells more sensitive to the cytotoxic agents. I believe that by working with the Cell Metastasis Group I will gain a better understanding of these processes and in the meantime will rely on advice from Dr. Cardelli. In any event, these studies have been removed and therapies will be addressed in a future R33 application. And finally, the reviewer comments on lack of publications, other than reviews. Unfortunately, coming from industry, our investors have not encouraged publishing data. We do have a published patent which is cited in the application and based on the research, Nucleonics recently closed on a $50M financing. Unfortunately, I can not directly address this concern.

I appreciate the third reviewer's comments that the identification of the pathways and mechanisms central to the process of metastasis and subsequent development of potential therapeutics is highly significant. While I have removed the therapeutic aspects of this proposal, as mentioned earlier, I still believe that RNAi represents a good therapeutic strategy and hope to pursue these activities in a subsequent R33 application.

I believe that this was a very fair review of the application and that by removing the synergy/therapeutic studies as suggested, the overall project is much more focused. The success of this project will not only result in the identification of novel targets for preventing cell metastasis but a general platform for identifying genes involved in other cancer related mechanisms and in other diseases.

A. Specific Aims

Hormonal therapies, cytotoxic agents and surgeries have been developed which improve survival of cancer patients but any given treatment results in only a moderate response rate in terms of absolute mortality, due primarily to the invasiveness of the primary tumor. In addition, these therapies are not particularly selective for tumor cells and are thus associated with severe side effects, including increasing risks of other types of cancer during hormonal treatment. Also, cytotoxic agents are of limited utility in the treatment of tumors expressing the multi-drug resistant (MDR) gene. Thus, there remains a tremendous need for new types of therapies and an associated need for new, well validated targets to which these therapies can be directed.

The purpose of this R21 application is to develop an innovative technology through the use of RNAi libraries to identify novel gene targets involved in cancer cell invasions. The platform, once developed, could also be used to identify genes involved in other attributes of cancer. Longer term, resulting RNAi molecules would further be used as a new therapy for the treatment of cancer. Specifically,

Aim 1. Develop stable cell line, expression systems and functional assay for RNAi library evaluation
   a. Create Flp-In plasmids for transfections, transfect and select stable MDA-MB-231 cells
   b. Develop functional assay for cell invasion
   c. *Develop a negative-selection assay for cell invasion*

Aim 2. Generate dsRNA library from invasive MDA-MB-231 cell line
   a. Select population of highly invasive cells from matrigel and isolate poly(A)+ mRNA
   b. Generate cDNA library using vector and poly(A)+ mRNA from above

Aim 3. Identify novel anti-invasive targets

a. Transfect library from Aim 2 into stable Flp-In cell lines developed in Aim 1 and select for stable recombinants
b. Isolate non-invasive clones by cell invasion assay
c. Identify sequences from non-invasive clone
d. Test RNAi sequences in MDA-MB-231 for prevention of cell invasion

The successful completion of the above Specific Aims will result in the development of a vector system which can be used to generate dsRNA libraries for evaluation of functional targets; a dsRNA library from a breast cancer cell line that can be used to analyze other functionally important cancer phenotypes and a technology to identify genes involved in cell invasion from other types of cancer; and novel, validated therapeutics targets for the prevention of breast cancer cell invasion.

The identification of genes involved in cell invasion through the use of RNAi libraries will provide targets that are critical for the invasiveness of the cancer but since inhibition of these targets will be non-lethal, should not be toxic and therefore be of use in combination with existing therapies. Also, since the resulting treatments will be comprised of RNAi molecules, MDR should not be an issue. The successful identification and cellular validation of such molecules in this application may lead to the development of novel RNAi therapeutics in a subsequent R33 application for the treatment of cancer. For RNAi molecules to be developed as successful therapeutics, further advances in in vivo delivery must occur. This type of work is ongoing at many of the biotechnology companies, for example Nucleonics, Benetic and Intradigm. In addition, the development of functional RNAi library silencing will have applications to the study of a variety of different cancer phenotypes. The ability to identify, validate and potentially develop therapeutic molecules all from the initial starting material (libraries of dsRNA) represents a tremendous advantage of this technology over other functional genomic approaches.

B. Background and Significance

With the advancement of early detection and treatment strategies, the 5 year-survival rate for breast, prostate and many other cancer patients is now at or near 90%[i]. For example, the use of gene expression array technology has not only allowed for the identification of many genes associated with breast cancer, but has also linked their expression pattern to prognosis[ii,iii]. However, when cancer cells metastasize by migrating from the primary tumor and invade other sites, the prognosis is much poorer. The understanding of how a primary tumor cell migrates from the initial tumor, disperses throughout the body in the vascular system, invades a new site and begins to proliferate and form secondary tumors in not well understood[iv], although it is well accepted that the mechanisms leading to metastases will represent attractive therapeutic targets[v]. Since migration and invasion are key components of cellular metastasis[5,vi], these processes have been well studied in cell culture systems and represent an attractive target for drug discovery.

For a cell to separate from the tumor and invade another cell mass, the proteins involved in extracellular matrix formation must first be altered. This was confirmed by Gordon *et al.* who showed that as cell lines become more invasive, they display more of a myoepithelial phenotype including changes in many extracellular proteins and an increase in matrix metalloprotease-7 (MMP-7), a proteolytic enzyme with broad-spectrum activity[vii]. The increase in expression of enzymes, such as the MMPs, during metastasis is likely to be essential in breaking down the extracellular matrices both at the sight of the primary tumor and the site of invasion and thus have been well studied. TACE, another metalloprotease was also demonstrated to play a key role in promoting migration and invasion[viii] and an inducer of the MMPs, EMMPRIN, was shown to be elevated in multidrug resistant cancer cells, with a corresponding increase in MMP-1, -2, and -9[ix]. COX-2, the target of thalidomide, a drug approved for the treatment of multiple myeloma and currently in clinical trials for a variety of other cancer indications[x,xi], increased cell invasiveness and MMP-2 levels when transfected into colon cancer cells[xii] and inhibitors of COX-2 reversed this invasiveness[10,xiii]. In addition, cystatin M, a protease inhibitor was found to be down-regulated in metastatic cells and its expression was able to block cell migration and matrix invasion[xiv]. Unfortunately, for all the promise the MMPs have shown in cell and animal models of invasion, MMP inhibitors have not faired well in clinical trials, often producing side-effects not seen in animals[xv]. Thus, additional targets for the prevention of cell migration and/or invasion will need to be studied before other classes of therapeutics can be advanced.

Other molecular targets previously linked to cancer, such as G-protein coupled receptors[6], IL-8[xvi], Rho C[xvii], the calcium binding protein S100A4[xviii], the ErbB receptor family[6,xix,xx], thromboxane synthetase[xxi], and the phosphoinositide 3-kinase/Akt[xxii] have all recently been shown to be associated with increased cell migration

and/or invasiveness. Molecular profiling by RNA expression arrays[xxiii,xxiv] and microsattelite instability of cell clusters[xxv] has resulted in the identification of a large number of potential targets associated with cell migration and invasion. However, many of these targets, particularly in the case of the molecular profiling studies, have not been validated as good, druggable targets – targets, which when inhibited will prevent cell migration and/or invasion but which will not be toxic to normal cells or cause side effects *in vivo*. Two recent reports suggests that retinoic acid[xxvi] and parthenolide[xxvii], an inhibitor of NF-□B, inhibit cancer cell invasion, however, neither retinoic acid nor NF-κB inhibitors have enjoyed tremendous success clinically as anticancer treatments although both have been investigated for many years[xxviii]. Coumarin analogs have also been shown to be capable of inhibiting cell invasion *in vitro* but their mechanism of action is unclear[xxix].

Antisense and short interfering RNAs (siRNAs) have been used to validate the function of numerous genes in cancer cell migration and invasion. Antisense molecules targeting *Id*-1[xxx], inhibitors of DNA binding which had previously been shown to be correlated with high invasiveness, prevented MDA-231 invasion *in vitro* and resulted in fewer lung tumors in nude mice. Cell invasion was also prevented when antisense against AKT2[xxxi], IGF-R1[xxxii] and eIF-4e[xxxiii], other targets that had previously been shown to be upregulated in cancer cells were studied. While encouraging, these studies all required prior knowledge of the target's role in cell migration and/or invasion and are therefore not amenable to the identification of new and perhaps better, therapeutic targets for the inhibition of migration and/or invasion. To identify such target, functional genomic approaches must be employed.

In addition to the gene array approaches[21,22] used to identify new targets for cancer cell migration and invasion, more recent proteomic strategies have also been employed for the analysis of breast cancer cell lines[xxxiv] and human breast cancer tissue[xxxv]. Proteomic approaches offer the advantage that the identified targets are actually expressed as proteins but like all genomic approaches, do not answer the question as to which of the altered proteins are important to the migration and invasion phenotypes as opposed to those that are a consequence of the important changes. The advent of functional genomics has sought to address the cause versus consequence issue by coupling molecular genetics to phenotypic changes. The earliest functional genomic studies involved transfecting an expression cDNA library from a cancer cell line into a non-cancerous cell and looking for genes allowing for phenotypes associated with cancer[xxxvi,xxxvii], such as growth in soft agar. The problem with such studies is that although a gene may be capable of causing a change in cellular growth characteristics, such genes are not necessarily those that are actually involved in these characteristics in human cancers. An alternative strategy is to transfect a cDNA expression library from normal cells into cancerous cells and look for changes due to the gain in function, such as the expression of a tumor suppressor gene[xxxviii,xxxix]. There is evidence that some tumor suppressor genes are involved in invasive potential[xl] but since invasion is secondary to tumor formation, it is more likely that loss of function will lead to migration and invasion. In addition, drug discovery efforts have generally been far more productive in identifying antagonist than agonist, thus given the evidence discussed above that over-expression of specific proteins can play a role in migration and/or invasion, the focus of this program will be on identifying promoters of migration and invasion so that antagonist can be developed as therapeutics. However, the RNAi functional library approach described can just as easily be used to identify migration/invasion suppressors by creating silencing libraries from invasive cells, transfecting them into non-invasive cells and assaying for gain of function.

Whole animal gene knockout studies have been successfully used to study gene function by companies such as Lexicon Genetics (www.lexicongenetics.com) and Deltagen (www.deltagen.com), but for the purposes of studying secondary processes such as migration and invasion have not been very successful since not only must a tumor form in the animal, but it must also metastasize and it is unlikely that a single gene will be responsible for both activities. Thus, *in vitro* cell knock out studies represent the best strategy for linking the loss of function of a gene in a tumor cell to migration and/or invasion.

Until recently these studies have involved the use of antisense or ribozyme RNA expression libraries. As described above, antisense molecules have been used to successfully study cell migration and invasion[30-33]. The mechanism of action is that through Watson-Crick base-pairing an RNA/DNA hybrid is formed which is then degraded by RNaseH. Libraries of antisense molecules have been used to study tumor suppression[xli] and Gentrove, a division of ISIS Pharmaceuticals, Inc. (www.isip.com), is wholly focused on validating targets and pathways using antisense technology. Chemistries affecting the stability and delivery of antisense molecules have been well studied and optimized[xlii,xliii]. These advances have led to the progression of antisense oligonucleotides into clinical trials targeting various cancers[xliv,xlv]. However, clinical trials of antisense oligonucleotides have been disappointing despite promising preclinical results[xlvi]. Perhaps the last hope for this class of molecules for the treatment of cancer is Genasense, an antisense oligonucleotide targeting Bcl-2[xlvii,xlviii], which is waiting approval from the FDA for treatment of malignant melanoma.

The lack of success of antisense molecules in clinical trials may relate to the fact that stochiometric concentrations of molecules are needed to inhibit gene expression since once the oligonucleotide binds to its target RNA, both are degraded. In addition, there are significant off-target effects of these oligonucleotides. Since new RNA is being transcribed, delivery of oligonucleotides must be maintained over time at levels sufficient to result in its degradation without affecting other targets, representing still another hurdle for their use therapeutically. Ribozymes overcome these limitations in that they are catalytic and specific. Libraries of ribozymes have been generated by inserting random sequences in the variable region of the molecule, transfecting the library and observing changes in phenotype[xlix,l]. A similar strategy of creating a library of hammerhead ribozymes has been used to identify genes involved in a cellular model of invasion[li] and in animals to demonstrate the role of NF-κB in tumor invasion[lii]. Unfortunately, similar to antisense, the therapeutic promise based on cell culture and animal studies has not been translated to clinical success, with one company (Ribozyme Pharmaceuticals, Inc.) abandoning its clinical trials and shifting its research interest to siRNA (in fact, the company has now been renamed Sirna) and the other company focused on ribozyme therapeutics (Immusol, Inc.) exploiting local rather than systemic diseases. Like antisense, delivery is a key issue, limiting the utility of this class of molecules in the clinic.

RNAi may yet offer the opportunity to create nucleic acid based therapeutics since, at least in non-mammalian systems, RNAi acts systemically[liii]. RNAi was originally described in plants, where it was believed to play a key role in protection against viral pathogens. The pathway involves a dsRNA of greater than 21 base pairs triggering an RNaseIII-like enzyme called Dicer[liv]. Dicer cleaves long dsRNAs, into small interfering RNAs (siRNAs) of 21-25 base pairs. These siRNAs then are incorporated into a multi-subunit RNA-induced silencing complex (RISC), which acts catalytically to target degradation of cellular mRNA in a sequence dependent manner[lv,lvi]. Given the systemic and catalytic nature of RNAi, this class of molecules has been proposed for use both in target identification/validation and the development of therapeutics[lvii,lviii].

Recently, an siRNA to c-met has been identified and shown to lead to apoptosis and reduction of tumor growth[lix]. In addition, when c-met is was knock-down using ribozymes, cell invasion and metastasis were inhibited both in vitro and in mice[lx,lxi]. A number of groups are targeting c-met for therapeutic intervention with small molecules[lxii,lxiii] given its important role in tumor metastasis [reviewed in lxiv]. For these reason, c-met will be used in the present studies as a positive control dsRNA for cell invasion.

Unlike nonmammalian species, long dsRNAs activate the dsRNA dependent protein kinase and interferon pathways[55,lxv], which can lead to general translational inhibition and apoptosis. This has led to an emphasis on the use of siRNAs. Libraries of siRNA molecules targeting a specific gene can be produced synthetically, although new enzymatic approaches[lxvi] have reduced the time and costs associated with producing libraries, and allow for the selection of the siRNA with the highest level of activity against the target of interest. Functional RNAi libraries have been designed and synthesized to study cytoskeleton organization in *Drosophila*[lxvii] and are being used extensively to study gene function in *C. elegans* [for review see lxviii] but have only recently been described for use in mammalian systems[lxix]. Blau's laboratory showed the ability to generate functional libraries of siRNAs against specific targets and the ability to generate a complex, 415,000 member siRNA library from an existing cDNA library but in the latter example, did not report that this library was functional. The authors go on to point out that they generate on average 34 unique siRNA clones per kilobase of sequence, a distinct advantage since not all siRNAs are active. However, this also points out the limitations of generating siRNA libraries. Significantly larger libraries will be required to ensure that active siRNAs are present for each transcript. Other approaches have recently been undertaken to develop functional siRNA libraries. Bernards' laboratory constructed a human shRNA library against 7,914 human genes believed to be involved in proliferation and upon screening the library identified 1 known and 5 novel modulators of p53-dependent proliferation arrest[lxx]. Shultz's lab has used a similar approach of creating a library of siRNA molecules in plasmids from 8,000 known genes to identify novel genes involved in NF-κB signaling[lxxi]. While these recent publications have validated the approach of making functional RNAi libraries to understand fundamental cellular mechanisms, they both require information and some knowledge of existing sequences since the RNAi sequences are generated synthetically. In addition, by not controlling for the number of plasmids taken up by a given cell, off-targeting effects can complicate the interpretation of the data[lxxii]. The focus of this application is to pursue the use of expressed long inhibitory RNA (eliRNA) libraries for identification of gene function by identifying efficient promoter systems, ensuring single copy integration, avoiding the interferon response and having a system that can be directly used in animals. Activation of the interferon response by siRNAs has also been described[lxxiii] thus this issue may not be unique to long dsRNAs and should not present insurmountable problems in developing and using the random library described in this application. By utilizing eliRNA, the cell will select the best siRNA molecule, increasing the complexity of the library while reducing the actual number of members needed for evaluation.

While the development of such a library will have broad applications for screening genes involved in various cancer phenotypes, the focus of this application will be on migration and/or invasion. Cell migration and invasion have been studied using siRNAs against specific targets, validating the role of these targets in migration and invasion[lxxiv,lxxv,lxxvi]. As described above, a great deal of effort has gone into studies to identify small molecules capable of preventing cell invasion and metastasis in vivo. While the molecules do appear capable of inhibiting the activity of their targets, they have not had the desired therapeutic activities. This suggests that additional targets must be identified or that strategies combining different treatment regime should be employed. The alternative, simply giving up on identifying therapeutic inhibitors of invasion/metastasis is unacceptable. The identification and validation of novel genes in this proposal critical to this pathway will provide the necessary information for the direct development of new therapies – that is, the isolated eliRNA clone, after further *in vitro* validation, will be used directly in animals and may ultimately become the therapeutic molecule. A number of companies, Sirna (www.sirna.com), Nucleonics (www.nucleonicsinc.com), Accuity (www.accuitypharma.com), Benitec (www.benitec.com.au) and Alnylam (www.alnylam.com) have anti-viral, macular degeneration and anti-cancer RNAi products moving toward clinical development.

C. Technical Vision/Preliminary Studies

1. Technical Vision

As discussed above, RNAi holds tremendous promise not only for target identification and validation but perhaps more importantly, as a new class of therapeutics allowing for the treatment of diseases for which current strategies have been unsuccessful[lxxvii]. The coupling of identification, validation and discovery should shorten the over-all drug discovery process by years, providing for more rapid introduction of new molecules into the drug development pipeline. However, for this process to be fully realized RNAi libraries must be screened instead of the current process of individually validating specific targets. The current approach requires some information about the target, thus novel but important targets may be missed. To accomplish this goal, there exist several hurdles which first must be overcome:

- Identification of a promoter system to ensure levels high enough to induce specific silencing without eliciting off-target silencing
- Identification of a system that can be used to generate single copy introduction of the eliRNA library for target screening
- Use of a vector system that can have direct clinical application
- Generation of a library for which all targets are well represented
- Use of eliRNAs in mammalian cells without inducing the interferon response

As these hurdles are overcome, a broadly applicable technology will be developed and the parameters will be established for using eliRNAs as therapeutics. Having a system available to generate additional libraries will provide a means of rapid target identification and validation for any analyzable phenotype, including anchorage-independent growth, isolation of anti-apoptotic proteins when screened in the presence of a compound like camptothecin and genes involved in growth regulation, in addition to genes involved in other diseases. *A longer-term* goal is to generate a system whereby eliRNAs can be used therapeutically. The ability to use long dsRNA would have significant advantages over siRNA, especially in treating viral disorders where point mutations in the viral genome may lead to escape from specific siRNAs but are unlikely to be tolerated over the length of a long dsRNA. Long dsRNAs have been used effectively to silence expression in mammalian cells[lxxviii,lxxix,lxxx], however additional work is required to ensure that routine use can be carried out in the absence of activation of the interferon pathway. For this to be realized, significant advances in delivery must be made, a problem which is being addressed by many of the companies mentioned above.

For this particular R21 application, the key milestone is that by developing this technology, eliRNAs can be identified that play a critical role in migration and/or invasion. By demonstrating efficacy of this approach in identifying new targets involved in invasion and metastasis, this platform could be used to screen for any functional effect and is not limited to known sequences as are current RNAi library strategies[64,65]. Future studies will include the thorough evaluation of the activity of these eliRNAs against a number of breast cancer and normal tissue cell lines and once activity is demonstrated, combined with a lack of toxicity, including the lack of induction of an interferon response, in all cell lines, the isolated eliRNA molecules will be well-positioned for testing in animal efficacy models. If the development of delivery systems for in vivo expression of RNAi molecules advances as expected, those that demonstrate activity, can be further developed through

preclinical. These cellular and animal efficacy studies and potential preclinical development work is projected to be carried out under a subsequent R33 application.

The expected outcome of this work would include:

- Development of an RNAi library target identification/validation technology
- The identification of novel therapeutic targets involved in cancer cell migration and/or invasion

Even in the absence of therapeutic development of eliRNAs, the proposed technology will provide for the rapid identification and validation of new anti-cancer targets. Once fully validated, these targets can be used in classical small molecule drug discovery efforts. As discussed above, the lack of effective therapeutics against existing targets requires a broader, non-biased screening approach to continue in the search for good therapies.

2. Preliminary Studies

Prior Experience of the Principal Investigator

The Principal Investigator on this application, Dr. Tony Giordano, has significant experience in the construction of cDNA libraries and the cloning of cDNAs, including EF-Iα[lxxxi], cytochrome oxidase II[lxxxii], pituitary glycoprotein hormone[lxxxiii], nicotinic acetycholine receptors[lxxxiv,lxxxv] and glutamate receptors[lxxxvi]. In the course of these studies, he has worked with phage, plasmid and cosmid libraries and has used transfection techniques including calcium phosphate, electroporation and liposome mediated strategies to create stable cell lines.

As head of research at Message Pharmaceuticals, Dr. Giordano oversaw the growth of four drug discovery programs, including an anti-Her2 program. Each of these programs involved identifying novel post-transcriptionally regulated RNA targets, setting up small molecule screens and validating compounds identified through the screening program. At the time he left Message, his research programs were involved with two corporate collaborations, molecules from three programs were entering animal studies and positive data was available from its antibacterial program against resistant staph. During the time he was at Message, he was an inventor on 10 patents, 5 of which have been granted or issued.

Dr. Giordano left Message to co-found Nucleonics, Inc. where he served as the President of the Company and was an inventor on one of the Company's key patents[69]. While heading up the company, Nucleonics focused on validating the use of long dsRNA for target validation. Initial work focused on overcoming the stress response.

As discussed above, mRNAs related in sequence to the dsRNAs are targeted for degradation. Long dsRNAs are processed intracellular to form siRNAs. Although long dsRNAs have distinct advantages, including better efficiency and efficacy, their link to the interferon response has limited their use in mammalian systems[lxxxvii,lxxxviii]. A number of parameters can lead to the induction of the interferon response, including structure, concentration and method of delivery of the dsRNA[lxxxix,xc,xci]. The initial objective at Nucleonics was to identify method(s) by which long dsRNA could be used to efficiently induce PTGS in mammals without invoking the Type I interferon stress response pathway.

Preliminary analysis using the STAT activation assay with human rhabdomyosarcoma (RD) cells, demonstrated that they possessed a functional type I interferon pathway (Figure 1). These cells were then used to analyze the induction of interferon by different delivery systems and different dsRNAs.

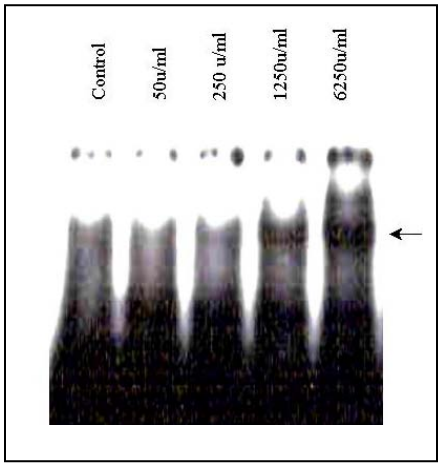


Figure 1: Human rhabdomyosarcoma (RD) cells are type 1 interferon responsive. RD cells were cultured to confluency in DMEM supplemented with 10%FCS. The medium was removed and replaced with DMEM containing different amounts of recombinant human IFN-α as indicated and incubated at 37 °C for 15 minutes. Nuclear extracts were prepared and 5 μg of each nuclear extract was incubated with ^{32}P-labeled SIE probe (contains consensus binding site for activated STAT 1 and STAT 3), and the reaction fractionated by polyacrylamide gel electrophoresis. The gel was then dried on a Whatmann filter and exposed to a Kodak phospho-storage screen and analyzed by Quantity One™ (Bio-Rad). The arrow indicates the gel shifted SIE probe. Supershift analysis demonstrated that this gel-shifted complex contains STAT 1 (data not shown). Lane 1: control, no interferon added; lanes 2-5: represent IFN-α stimulation at 50u/ml, 250u/ml, 1250 u/ml and 6250u/ml respectively. These results demonstrate the ability of RD cells to activate STAT in response to IFN-α stimulation. Similar results were obtained for beta-interferon stimulation (data not shown).

Various types of RNA delivered to/expressed in rhabdomyosarcoma (RD) cells were assayed for their ability to induce/activate key components of the Type 1 interferon response. Briefly, poly (I)(C) RNA, in vitro transcribed dsRNA of 600bp and in vitro transcribed ssRNA of 600bp were delivered to RD cells as naked RNAs or as complexes comprised of cationic lipids. siRNAs were also delivered to cells as cationic lipid/RNA complexes. In the particular experiments presented here, the cationic liposome, lipofectamine (Invitrogen), was used. RNA/cationic lipid complexes were all made using identical charge:charge ratios. dsRNA was also expressed intracellularly using the T7 RNA polymerase expression system.

The following parameters of the Type 1 interferon response were monitored following delivery/expression of RNA to/in RD cells: alpha/beta interferon production, 2'5'-OAS mRNA induction and PKR and 2'5'-OAS activation. Cytotoxicity was also evaluated through the use of an apoptotic nuclear staining assay (TdT FragEL, DNA Fragmentation Detection Kit, *In Situ* Apoptosis Assay from Oncogene (Boston, MA)), the measurement of antiproliferative responses and the visual recording of cytopathic effect. For interferon analysis, supernatants were removed from RNA stimulated and control cells at various times points. Interferon-alpha and beta were measured using the human interferon-alpha ELISA kit from Endogen (Rockford, IL) and the human interferon-beta ELISA kit from RD1 (Flanders, NJ) according to manufacturer's directions. The detection of mRNAs encoding the p69 human 2',5'-oligoadenylate synthetase was performed by reverse transcriptase PCR using the Titan One Tube Reverse transcriptase PCR Kit (Roche Biochemicals, Nutley, NJ) according to the manufacturer's directions. Primers and conditions for the p69 encoding mRNA were as in Hovnanian et al. PKR activation was monitored by measuring the ratios of phosphorylated to non-phosphorylated eIF2alpha using Western blot analysis and antibodies specific for phosphorylated eIF2a and non-phosphorylated eIF2a. Measurements were made at various time points over 24 hours following RNA stimulation. Since activation of PKR was found to peak at different times (dependent on the RNA delivered), and since the ratio of phosphorylated to unphosphorylated eIF2alpha changes (in control cells) over 24 hours, each sample was compared to the appropriate time point control and expressed as fold over control. 2'5'OAS activation was monitored using a ribosomal fragmentation assay. Figure 2 summarizes the data from these experiments. No interferon alpha was induced by any of the treatments, while similar levels of beta-interferon were made by cells exposed to cationic lipid complexes containing poly(I)(C), and in vitro transcribed dsRNA (600bp) or ssRNA (600nts). The presence of beta-interferon was correlated with 2'5' OAS mRNA induction, however activation of 2'5' OAS was only seen in cells exposed to those complexes containing either poly(I)(C) or dsRNA. In conclusion, cytoplasmic expression of dsRNA was the only method tested found to completely avoid induction/activation of the RNA stress response pathway as measured by the parameters that were studied.

Summary of Stress Response Studies

Effector	IF-alpha	IF-beta	Cytotoxicity	Peak eIF2α-P (Control =1)	2'-5'OAS Ind./Act.
Naked:					
Poly(IC)	-	-	+	ND	-/-
dsRNA (600 nts)	-	-	-	ND	-/-
ssRNA	-	-	-	ND	-/-
CL Complexed					
Poly(IC)	-	+	+++++	1.8	+/+
dsRNA (600 nts)	-	+	+++	3.0	+/+
dsRNA + DNA	ND	ND	+++++	ND	ND
ssRNA	-	+	-	1.1	+/-
dsRibo-oligo(si RNA)	-	-	++	1.5	ND
CL Complexed DNA Intracellularly Transcribed dsRNA	-	-	-	1.05	-/-

Figure 2: Summary of RNA stress response studies in RD cells. Following RNA administration/expression as described in text, RD cells were assayed for various components associated with a type I interferon response. The symbols are as follows: + (detectable response), - (no detectable response) and ND (not determined). The relative levels of cytotoxicity are indicated by the number of + symbols, for example cationic lipid complexes containing poly(I)(C) were more cytotoxic than those complexes containing in vitro transcribed dsRNA. Peak eIF2alpha-phosphorylated levels are designated as fold over control cell levels of phosphorylated eIF2alpha.

Once the ability to delivery and express long dsRNAs without inducing the stress response in mammalian cells was demonstrated, a comparison of efficacy of long versus short dsRNA was carried out. The effectiveness and persistence with which siRNA and cytoplasmically expressed long RNA can induce gene-specific silencing was compared in a transient SEAP (secreted human placental alkaline phosphatase) expression system. RD cells transiently expressing SEAP were transfected either with one of three different SEAP-specific siRNAs or with a T7 RNA polymerase expression plasmid in conjunction with a SEAP-specific dsRNA expression. As an internal control for silencing specificity, the cells used in these studies also transiently expressed secreted murine IL-12. Media collected from transfected cells at multiple time points

post-transfection was assayed for both SEAP and IL-12. SEAP expression levels were normalized to IL-12 levels.

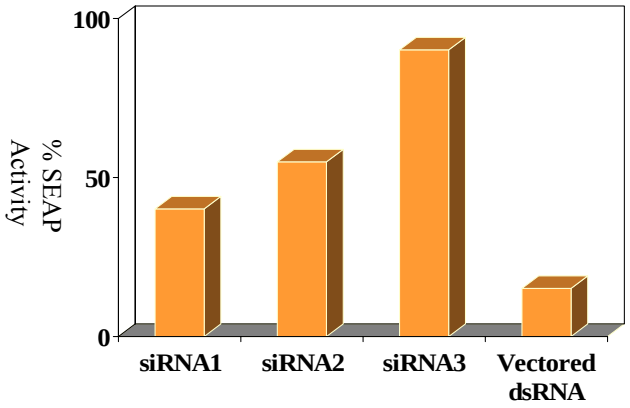


Figure 3: Comparison of SEAP specific siRNAs and intracellular expression of long SEAP specific dsRNA. RD cells were transfected with pHCMV-SEAP and pHCMV muIL-12 and co- transfected with 5 µg of three different siRNAs. Alternatively, cells were co-transfected with a SEAP dsRNA expression vector and a T7 RNA polymerase expression vector. The total amount of nucleic acid was held constant at 5 ug per transfection, using an inert filler DNA where needed. SEAP levels were measured in the media 3 days post-transfection.

The three different siRNAs evaluated in this study were found to have different efficacies with respect to their ability to silence SEAP expression. siRNA #1 was the most potent, inhibiting SEAP expression by about 80% for two days. At a lower dose of this siRNA was less effective and maximal inhibition of SEAP expression was observed for only one day. siRNA #2 was moderately effective when given in the highest dose, resulting in 70% inhibition of SEAP that lasted for one day. When given at the lower dose, this siRNA resulted in a 40% inhibition of SEAP expression that persisted for two days. The least potent of the siRNAs, siRNA #3, resulted in a short-lived 55% inhibition when the siRNA was administered at the highest dose, that was completely abolished by day 3. SEAP activity on day is shown in Figure 3. All siRNA-mediated inhibition was lost between days four and six in these studies. In contrast, the use of the T7 cytoplasmic transcription system to generate long dsRNA in the cytoplasm of transfected cells was found to induce an 85% inhibition of SEAP expression that persisted for the length of the experiment, ten days. A dose response experiment in which different amounts of the best siRNA (#1) and the expression vector were transfected into RD cells and SEAP levels were monitored over six days was carried out (Figure 4). As described above, the silencing effect generated by the siRNA was transient and the maximal effect is dependent on the concentration of the siRNA molecule added to the culture. However, even at high concentrations, the level of silencing is less than that observed for the eliRNA. A control dsRNA expression vector encoding an irrelevant dsRNA had no effect on SEAP expression. The observed long-lived silencing response in this experiment is consistent with the continued expression of dsRNA that this expression system affords.

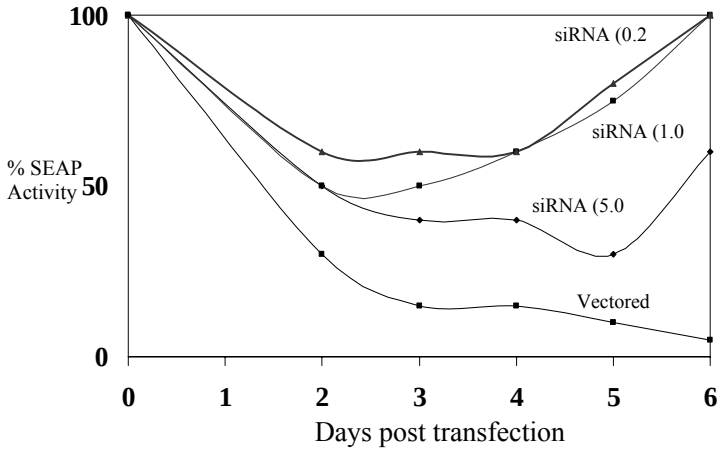


Figure 4: Dose response to increasing amounts of transfected (0.2, 1 or 5 ug) siRNA #1 or the SEAP dsRNA expression vector (vectored or eliRNA). SEAP was measured in the media of transfected cells at 48, 72 and 89 hours post-transfection.

After Dr. Giordano left the Company, Nucleonics shifted focus to anti-viral drug discovery and is no longer working in the target identification/validation area. However, this early work provides for the basis of the present application.

Prior Experience of the Research Scientist

Dr. Tada Utsuki received his post-doctoral training at the School of Medicine in Johns Hopkins University (Baltimore, MD) and the Drug Design & Development Section of the National Institute on Aging

(NIA), National Institutes of Health (NIH). His research focused on the functions of therapeutic drugs for the treatment of neurodegenerative diseases (e.g., brain tumor, Alzheimer's disease (AD). His work contributed to the discovery and characterization of a compound currently in Phase III clinical trials.

In September 2000, Dr. Utsuki joined Message Pharmaceuticals (Malvern, PA) as a scientist to investigate the discovery of small molecules targeting post-transcriptional regulation of the amyloid precursor protein as potential AD therapeutic. He is named as a co-inventor on two U.S. patent applications related to novel therapeutic drugs for AD based on this work. In 2002, Dr. Utsuki extended his interest in post-transcriptional gene regulation and drug discovery to RNAi and took a position with Nucleonics, Inc.

At Nucleonics, his research focused on the application and characterization of RNAi activity *in vivo* by dsRNA derived from DNA vectors. Dr. Utsuki was responsible for carrying out studies in mice with the goal of developing an RNAi-based therapeutic. His initial work focused on silencing endogenous creatine kinase gene expression by intramuscular injection of a plasmid expressing dsRNA to creatine kinase. The plasmid expressing creatine kinase dsRNA lowered creatine kinase levels by more than 80% in the muscle compared to vehicle (Figure 5).

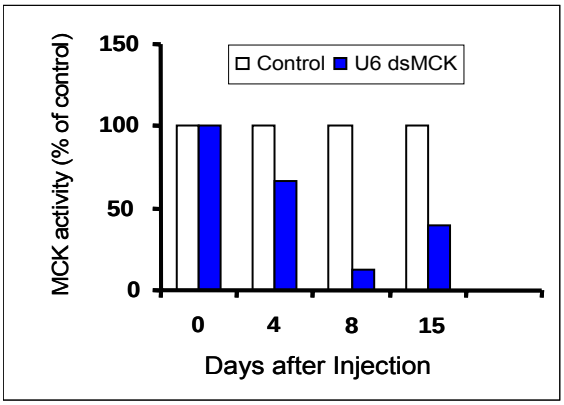


Figure 5: Effects of dsRNA *in vivo* 300 base pairs of the gene encoding creatine kinase was cloned downstream of a U6 promoter in the sense and antisense orientation in DNA plasmids. 10 μg in 100 μl of each plasmid prepared, formulated with physiological solvent (30 mM sodium citrate, 150 mM sodium chloride, 0.1%w/v EDTA) and injected intramuscularly in the left thigh of mice. 100 μl of vehicle was injected in right thigh of the same mouse as a control. The animals were sacrificed with CO_2 asphyxiation at the stated period (day 0, 4, 8 and 15) and muscle tissues were collected to examine various biochemical markers. Creatine kinase activities in mouse muscle were determined using the CK-NAC (UV) kit (Sigma) and normalized to control levels.

Recent Work Completed at LSUHSC-S

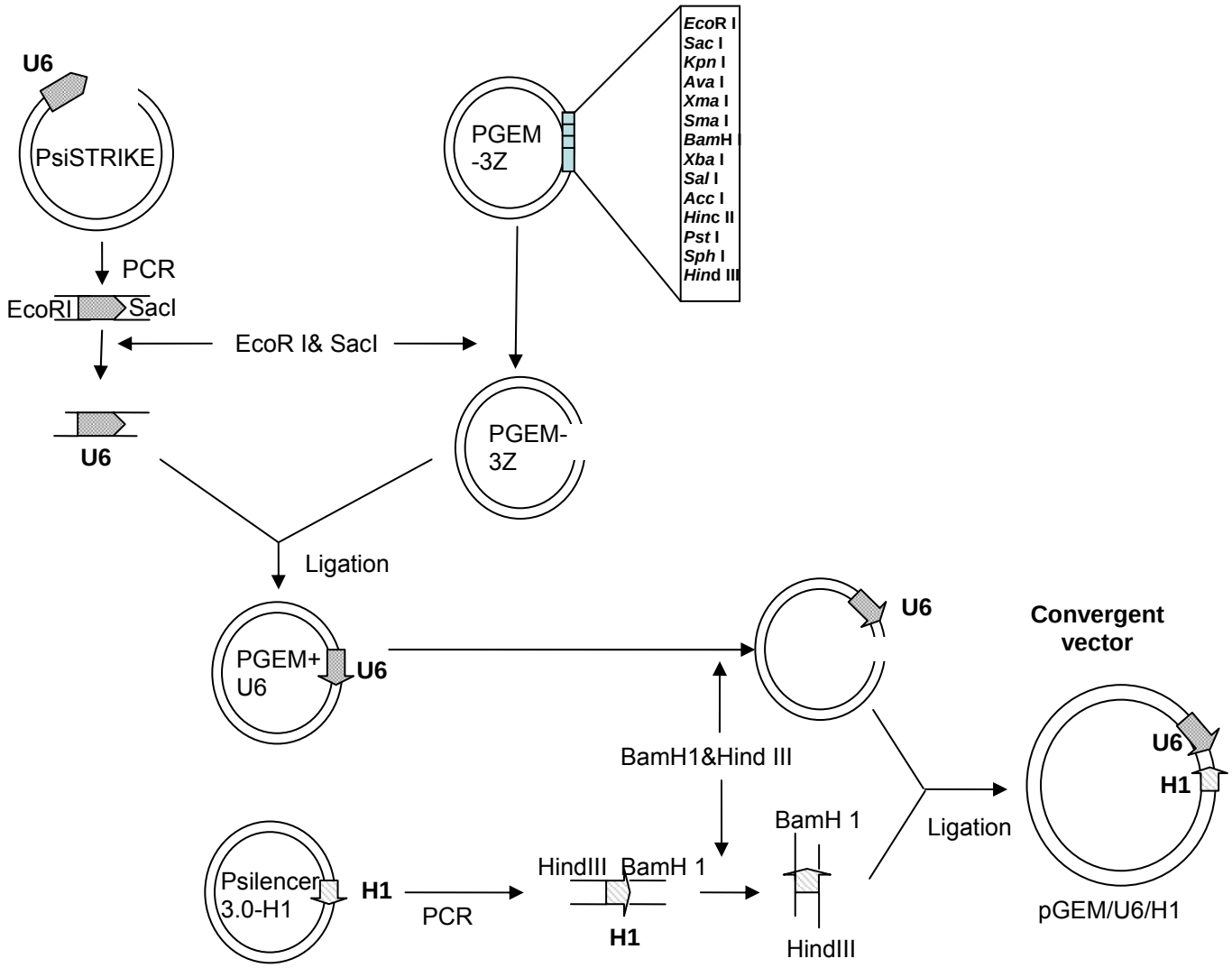
To create vectors containing dual, convergent promoters for expressing dsRNA molecules two different polIII promoters, U6 and H1, were cloned into pGEM. The scheme below was developed to create the construct. The primers used to PCR these promoters were designed to contain unique restriction sites at the 5' and 3' ends for cloning and a TTTTT stretch in the reverse primers to create the polIII termination signal:

EcoRI
U6 F: 5'GCCATGGAATTCAGAGGGCCTATTTCCCATG (Tm:63.8)
U6 R: 5'ATGTAAGAGCTCTTTTTCGGTGTTTCGTCCTTT CCAC (Tm:63.6)
SacI

HindIII
H1 F: 5' GCCGCGAAGCTTAATTCATATTTGC (Tm:57.4)
H1 R: 5' ATCTAAGGATCCTTTTTCGAGTGGTCTC (Tm:57.8)
BamHI

The polIII dual promoter construct has been confirmed by restriction analysis and is currently being sequenced. Following sequence confirmation, the following luciferase fragments will be cloned into the KpnI site in pGEM/U6/H1

FF 1-1000 F: 5'ATATAAGGTACCATGGAAGACGCCAAAAAC(Tm:58.8)
FF 1-1000 R: 5'ATATAAGGTACCGCAGATGGAACCTCTTG(Tm:59.4)
FF 1-250 R:5'ATATAAGGTACCGTTTTCACTGCATACGACG(Tm:59.7)
FF 250-500 F:5'ATATAAGGTACCACTCTCTTCAATTCTTTATGCC (Tm:57.9)
FF 250-500 R:5'ATATAAGGTACCGAACGTGTACATCGACTG(Tm:59.1)
FF 500-750 F:5'ATATAAGGTACCCGTCACATCTCATCTACCTC(Tm:59.7)
FF 500-750 R:5'ATATAAGGTACCGTAAACATTCCAAAACCGTG(Tm:58.6)
FF 750-1000 F: 5'ATATAAGGTACCACTACACTCGGATATTTGATATG(Tm:57.2)



This will allow for the cloning of 250, 500, 750 and 1,000 bp regions to determine size limitations along with multiple 250 bp sequences from different parts of the gene to identify whether different regions have different efficiency of silencing as described for siRNA. Each of these constructs will be tested against a FaDu cell line which has been generated in the laboratory to stably express firefly luciferase (10,000 units from 10^4 cells). As a positive control siRNAs obtained from Dharmacon and an shRNA generated using the original pSTRIKE plasmid and the Dharmacon siRNA sequence will be utilized.

Complexity of Cell Invasion and Rationale for Creation of dsRNA Libraries

To get a better understanding of the genes involved in cell invasion, a gene array analysis was carried out. MDA-MB-231 cells were placed in a Trans-well chamber and 10% fetal bovine serum was added to the bottom chamber as a chemo-attractant. Twenty-four hours later, cells were recovered from the top and bottom of the chamber and total RNA was isolated from each population and analyzed on Affymetrix Human Array 133A chips. A pairwise analysis of genes involved in cell migration identified 43 genes that were increased in the migrated cell population and 53 genes that were decreased (Figure 6).

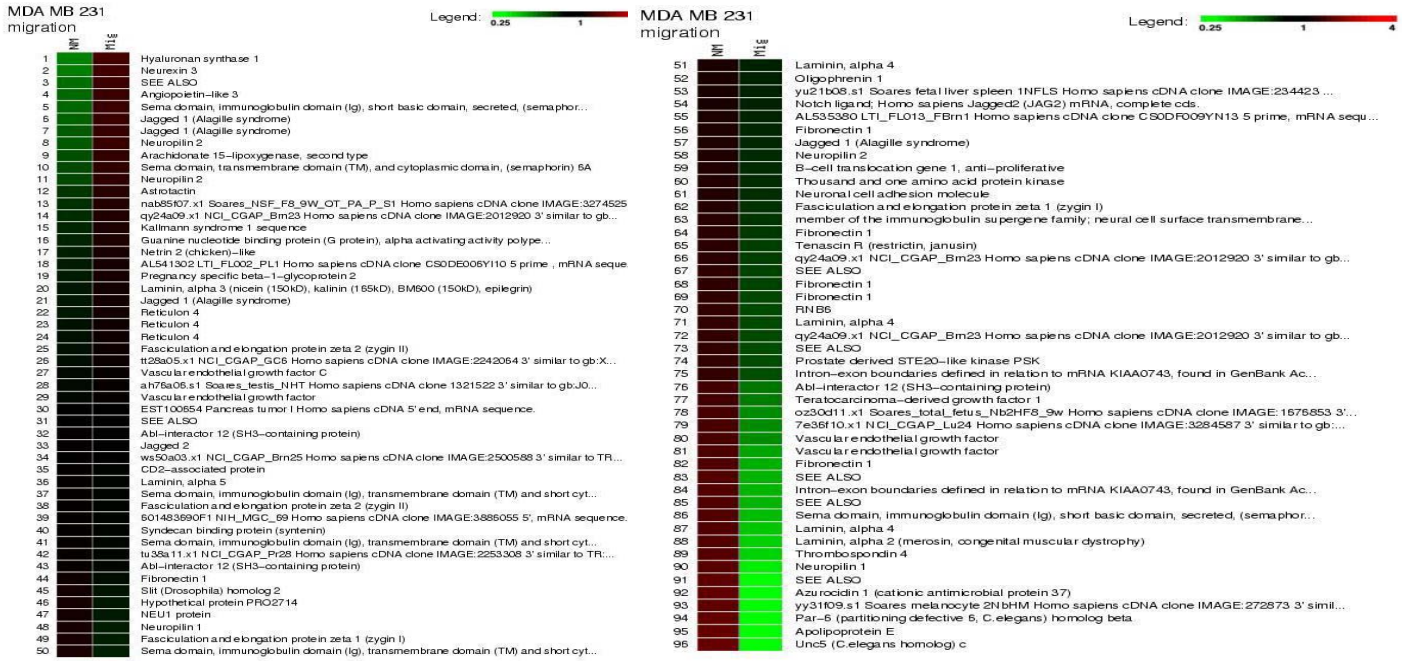


Figure 6: Pairwise analysis of genes involved in cell migration differentially expressed in MDA MB 231 invasive /non invasive.

Of those genes that are unique to one of the populations, 90% are specific to the invasive cells. These genes would represent good targets for silencing since they were not found in the population of cells that did not migrate and are thus potentially associated with cell migration but are not essential. However, it is likely that many of these genes are simply a consequence of migration and thus not necessarily functionally important. Interestingly, one gene known to be involved in invasion and of interest to us, c-met, was increased by close to 2-fold in the invasive cells. A limitation with this genomic approach is that changes in gene expression do not always correlate with changes in protein expression due to post-transcriptional regulation, particular differential protein synthesis. Thus, while some information may be gained by such an approach, it does not alleviate the need for functional assays.

<u>Luciferase Inhibition and Lack of Interferon Induction by pGem/U6/H1 driven dsRNA</u>

The dual polIII convergent promoter vector described above was used to determine the efficiency of silencing gene expression using long dsRNA cloned into an expression vector. Interferon induction was also monitored.

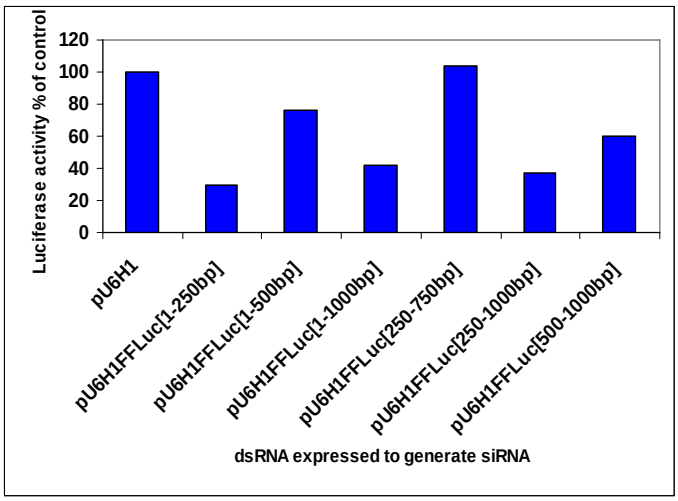


Figure 7. Firefly luciferase gene silencing in transiently transfected MDA MB231 cells with long dsRNA expressed from pGem/U6/H1 vector. The pU6H1 vectors above were co-transfected with pMS110 (luciferase driven by a RSV promoter) and luciferase activity was analyzed 48 hours later. Firefly luciferase activity was used as a read out and normalized to vector control.

Insertion of various fragment sizes of luciferase ranging from 250 to 1,000 base pairs resulted in the differential silencing of luciferase expression ranging from no silencing to 80% inhibition of luciferase activity (Figure 7). To determine whether these vectors induced an interferon response in MDA-MB-231 cells,

interferon beta activity was assayed. The results of this analysis, shown below, demonstrate a lack of interferon induction 48 hours after transfection at time at which efficient silencing has been observed.

MDA MD 231 FFLuc (MS110) 48h							
				AVERAGE	**STDEV**		**IFN-beta**
lipofectamine 0.25uL		0.044	0.036	0.04022219	0.0056295		-7.01341
pU6H1 45ng		0.046	0.040	0.04303695	0.0040903		-6.52811
pU6H1[1-250bp]1B 45ng		0.042	0.041	0.04130019	0.0002934		-6.82755
pU6H1[500-750bp]3B 45ng		0.045	0.043	0.04406913	0.0011551		-6.35015
pU6H1[1-500bp]4C 45ng		0.045	0.040	0.04235661	0.003244		-6.64541
pU6H1[1-500]4H 45ng		0.046	0.044	0.04476281	0.0011723		-6.23055
pU6H1[250-750bp]5A 45ng		0.043	0.044	0.04334506	0.0010805		-6.47499
pU6H1[250-750bp]5B 45ng		0.049	0.057	0.05304551	0.0059398		-4.8025
pU6H1[250-750bp]5F 45ng		0.046	0.046	0.04597244	0.0004377		-6.02199
pU6H1[500-1000bp]6A 45ng		0.048	0.047	0.04750944	0.0005914		-5.75699
pU6H1[500-1000bp]6G 45ng		0.052	0.048	0.04985284	0.003255		-5.35296
pU6H1[1-1000bp]8C 45ng		0.050	0.054	0.05186328	0.0025675		-5.00633
pU6H1[1-1000bp]8Abis 45ng		0.044	0.049	0.0467889	0.0037593		-5.88122
pU6H1[250-1000bp]9A 45ng		0.045	0.047	0.04599986	0.0012422		-6.01727
pU6H1[750-1000bp]10 45ng		0.047	0.055	0.05131025	0.005572		-5.10168
non treated		0.048	0.046	0.04732229	0.0013906		-5.78926

A similar construct was used to silence two endogenous genes, arc and NR1, in the PC12 cells. As in the above experiments, the 200+ nt dsRNAs each silenced their respective gene expression by 80% as analyzed by real-time PCR. In addition, a stable population and stable clones have been generated, further illustrating the lack of interferon activation, even with prolonged expression of dsRNA.

Preliminary Analysis of the Use of dsRNA in Cell Migration Assays

To analyze the ability of dsRNA for use in cell migration assays, a 250-bp insert of the c-met gene was cloned into the above dsRNA vector. C-met had previously been demonstrated to be involved in cell migration (see background section) and was found to be enriched by almost 2-fold in the population of migrating MD-MBA-231 cells from gene array data presented above. MDA-MB-231 cells that had previously been stably transfected with firefly luciferase were then transiently transfected with either a plasmid capable of producing dsRNA to c-met or to luciferase. Both transfections inhibited the targeted gene by greater than 50% (Figure 8).

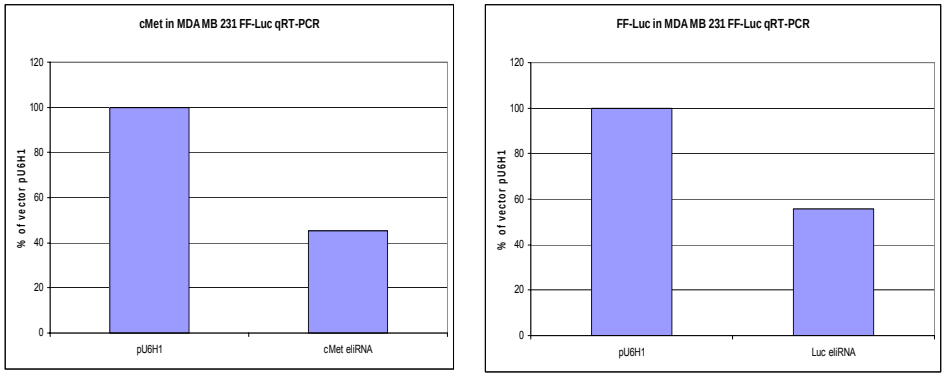


Figure 8. Cells were transiently transfected using Lipofectamine2000. 48 hours after transfection, total RNA was isolated using Trizol and levels of c-met and luciferase were quantified. For each experiment a vector without insert was used as a control..

The transiently transfected cells were then tested for their ability to inhibit cell migration using the transwell assay. The ability of expressed long c-met dsRNA to inhibit migration was compared to that of an siRNA that had been previously demonstrated to inhibit cell migration. As can be seen in Figure 9, both the expressed long dsRNA and the siRNA inhibited cell migration by ~40%.

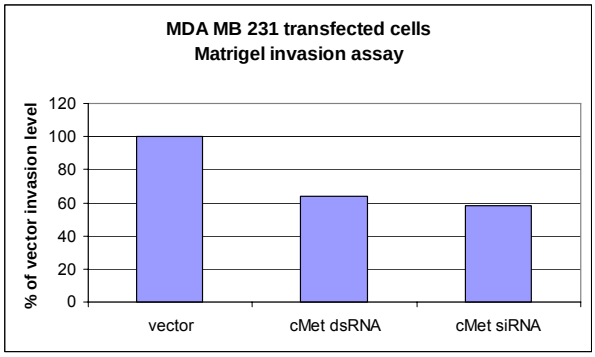


Figure 9: MDA-MB-231 cells were transiently transfected with either a plasmid encoding c-met long dsRNA or c-met siRNA (Dharmacon) by Lipofectamine 2000. Twenty-four hours after transfection, cells were added to a Transwell Chamber in matrigel and the number of cells entering matrigel were compared to those transfected with vector alone.

The inability to inhibit cell migration/invasion to higher levels is likely due to the inefficiency of transfection. In order to address this problem and to begin to analyze the issue of the robustness of the matrigel invasion assay, we have quantified the partitioning of the plasmids expressing long dsRNA between the migrated and non-migrated cells using real time PCR. Standard curves were generated using the plasmids encoding c-met and luciferase to quantify actual plasmid levels from isolated cells. As expected, luciderase plasmid was found in relatively similar levels in both the migrating and non-migrating cells, whereas the c-met plasmid strongly partitioned with the non-migrating population (Figure 10).

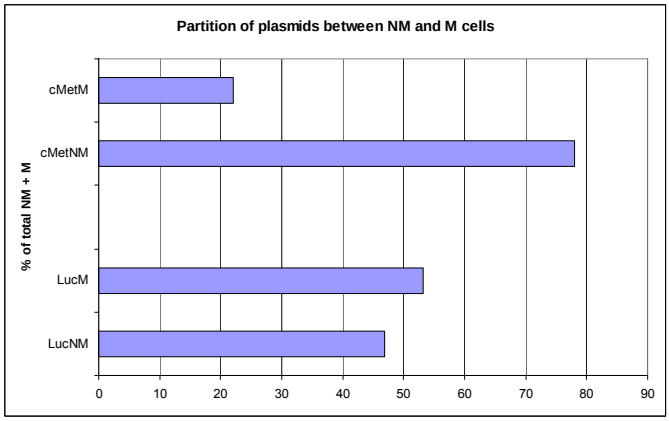


Figure 10. MDA-MB-231 cells were transiently transfected with a plasmid capable of generating either c-met or luciferase long dsRNA. Following a transient transfection, the cells were placed in a Transwell chamber containing matrigel and 24 hours later the migrated and non-migrated population of cells were isolated. DNA was recovered from each population and the levels of plasmid were analyzed using primers derived from the U6 and H1 promoters by qPCR using the ABI7700.

In total, these experiments present data that demonstrate (1) the ability to produce long dsRNA and efficiently silence gene expression when expressed by a plasmid in various cell lines; (2) the lack of interferon induction in various cell lines (MD-MBA-231 and PC12) and with various constructs (luciferase, c-met, arc and NR1) following transfection and efficient silencing; (3) the ability of a long dsRNA (c-met) to inhibit migration; and, (4) the partitioning of this plasmid (c-met) with the non-migrating cells since silencing of c-met would be expected to lead to prevention of migration. These preliminary experiments provide the basis for the following proposed studies.

D. Research Design and Methods/Milestones

1. Research Design

The main focus of this application is the development of a robust functional RNAi technology that can be used to identify and validate novel anti-cancer targets, the product of which (the RNAi used to identify the target) will eventually become the actual drug. For the strategy to succeed, an efficient expression system and delivery method must be identified such that the resulting large dsRNAs do not induce the stress response pathway. Thus, the first specific aim will focus on developing a system to express long dsRNAs without inducing an interferon response. In addition, the sensitivity of the functional assay will be analyzed.

Aim 1. Develop stable cell line, expression systems and functional assay for RNAi library evaluation

This series of studies will use both luciferase and *c-met* to study efficiency of silencing for rapid analysis (luciferase) and the ability to silence an endogenous gene (*c-met*). For the library strategy to be successful, each cell will take up a single plasmid. This will be accomplished using the Flp-In cloning system (Invitrogen). A similar strategy using Cre-loxP and siRNA has recently been reported to control expression of the siRNA[xcii] .

A stable Flp line will be generated for use in subsequent experiments ensuring equal plasmid numbers and limiting artifacts due to integration. In addition, various promoters will be analyzed to determine the most efficient means of silencing gene expression without inducing the interferon response. And finally, the cell invasion assay will be qualified to ensure the ability to detect and propagate the few cells for which invasion have been inhibited.

Create Flp-In plasmids for transfections, transfect and select stable MDA-MB-231 cells

Target cell lines will be created by using the Flp-In System (Invitrogen). Prior to transfection, a dose response curve will be carried out to determine the minimal concentration of zeocin required to kill the untransfected population of MDA-MB-231 cells. The cells will then be transfected using Lipofectamine2000 as described by the manufacturer's protocol (Invitrogen) with various concentrations of pFRT/*lac* zeo. Two days after transfection the cells will be trypsinized and replated at 25% confluence. After the cells have attached, the zeocin will be added at the concentration determined above. The cells will continue to be grown in media containing zeocin until foci form and individual foci will be transferred to a 96-well tissue culture plate. Recent generation of stable FaDu cells has demonstrated that approximately 50% of the selected colonies express the desired gene and that there is a distribution of 50% low expressors, 35% mid expressors and 15% high expressors. Thus, to ensure a similar distribution, 96 clones will be analyzed with the expectation that approximately 5 clones will be integrated in regions of the genome that allow high levels of expression. *The clonal cells will be expanded to greater than 10^7 cells each at which time genomic DNA will be extracted from 90% of each individual population, the genomic DNA will be digested with a restriction enzyme that does not digest the pFRT/lac zeo, run on a agarose and used for Southern analysis to determine the number of integrants by probing with labeled pFRT/lac zeo plasmid.* Clones with single integration sites will be further analyzed for maximal expression of β-galactosidase activity, with a minimal level that would result in a 10 fold signal to noise ratio, and continued ability to migrate across a collagen membrane to ensure that the site of integration did not affect the phenotype under study. The clone which demonstrates the highest expression levels and greatest migration potential will be used for the cloning of the RNAi library.

Once host cells are generated, the library can be readily cloned into a single site in the genome. The plasmid provided with the Flp-In system for this purpose contains a CMV promoter. The use of long dsRNAs and rapid library construction precludes the use of hairpins and therefore will require the use of dual promoters for simultaneous synthesis of sense and anti-sense strands of RNA. The optimal expression system will either be subcloned into the pcDNA5/FRT plasmid by replacing the CMV- BGH polyadenylation region *with the U6/H1 cloning region* or conversely, the FRT – hygromycin resistance gene can be subcloned into the vector selected below, based on the most efficient subcloning strategy available, i.e., convenient restriction sites and PCR product generation. For example, the CMV-BGH fragment can be excised by a double digest with *Spe*I and *Ban*II, blunt-ended with klenow and relegated followed by cloning of the dual promoter construct into the *Bgl*II site or the ~1,200 base pair FRT-hygromycin region can be cloned by PCR with unique restriction sites added to the primers for subsequent subcloning into the vector created below. Each would produce a vector allowing convergent transcription of RNAs and thus formation of dsRNA *in vivo*, selection of stable clones with hygromycin and single recombinants when co-transfected with pOG44 into the host cells. The stable, singly integrated library will then be analyzed for repression of migration and invasion as described below.

Given the perception that long dsRNAs will induce the interferon response in mammalian cells, essentially all the commercially available vectors, including the pSilencer series (Ambion), the psiSTRIKE series (Promega), and the Block-It vector (Invitrogen) utilize a single promoter, usually U6, and short hairpin RNAs. However, creation of a cDNA library precludes the use of hairpins since the complementary strand can not be readily cloned downstream of the hairpin sequence. Recent studies have successfully used dual, convergent polIII promoters to generate siRNAs. The U6-H1 strategy being employed in this project was used to generate a library of siRNAs from known genes to identify novel inhibitors of the NF-κB pathway in HEK and HeLa cells[65] and a similar plasmid (pHippy) was used to silence both a reporter and endogenous genes in HEK cells[xciii]. In addition, dual, convergent U6 promoters were used to silence gene expression in the liver of mice[xciv]. Although silencing was slightly more effective with the shRNA vector when compared to the dual promoters, the dual promoters still resulted in a significant ($p<0.01$) 75% decrease in luciferase expression in the liver. These studies clearly demonstrate the feasibility of using dual, convergent promoters. While these studies suggest that either the same polIII promoter[90] or different polIII promoters[65,89] can be used effectively to silence expression, the use of two different types of promoters will be employed in this project to prevent any possibility of recombination. An earlier study has also shown that the use of convergent promoters with siRNA can be an effective means of silencing gene expression for sustained periods in cultured cells[xcv] and thus the use of a similar strategy with long dsRNA should be feasible. *A vector has been developed and is described in*

Section C. This vector has been used to efficiently silence luciferase, c-met, arc and NR1 and two different cell lines (MDA-MB-231 and PC12) without inducing the interferon response. Adeno- and lenti-viral expression vectors represent additional alternatives, for which expertise exists at LSUHSC for their use. *In fact, the lenti-viral system with the same U6/H1 convergent promoters has also been successfully employed in our laboratory to silence arc and NR1 expression in both PC12 cells and primary neurons.*

Silencing will be monitored by using Promega's luciferase kit and the Rosys Anthos 96-well luminometer by plating cells at 10^4 cells per well and analyzing luciferase activity at 24 hours after transfection, and additional times out to two weeks for the time course assay. In addition, the stress response pathway will be assessed as carried out above. Interferon levels will be monitored by collecting supernatants from transfected and control cells at various times after transfection. The supernatants will be analyzed for the presence of human interferon alpha, beta and gamma. Interferon-alpha is measured using the human interferon-alpha ELISA kit from Endogen (Rockford, IL) according the manufacturer's directions. Interferon-beta is measured using the human interferon-beta ELISA kit from RD1 (Flanders, NJ) according to manufacturer's directions. Human interferon-gamma is measured using the interferon-gamma ELISA kit from R&D Systems (Minneapolis, MN) according to manufacturer's directions. The detection of the mRNA encoding the human 2',5'-oligoadenylate synthetase locus is performed by reverse transcriptase PCR using the Titan One Tube Reverse transcriptase PCR Kit (Roche Biochemicals, Nutley, NJ) according to the manufacturer's directions. Primers and conditions for the p40, p69 and p100 gene were as in Hovnanian *et al*.[xcvi] If activation with of this system with the long dsRNA constructs can not be overcome by altering promoters and/or transfection techniques, two alternative strategies will be undertaken. The first will be to generate smaller RNAi molecules by digesting with multiple 4 base pair cutters. This approach has the disadvantage that far more cells will need to be screened to obtain complete library representation. A second approach would be to use an siRNA to interferon to knock out this pathway in the MDA-MB231 cells. Clearly, resulting cells would have to be retested for continued metastatic activity.

Once parameters have been established concerning the best expression system and transfection method for optimal silencing of luciferase without inducing the stress response pathway, additional experiments will be carried out to confirm that this method is capable of silencing endogenous gene expression. *c-met* will be subcloned in 200-400 base pair fragments into the *Frt-U6/H1* vector, transfected into *MDA-MB-231 cells containing the integrated Flp-In site* and *the number of integrants will be monitored by restriction digestion and Southern analysis as described above. This system should produce single integrants, if additional integrants are observed, the amount of traansfected plasmid will be titrated down until only the correct integrant is found. c-met RNA and* protein *levels will be* analyzed by *real-time PCR and* western analysis using *c-met* antibody (Santa Cruz) and HRP-conjugated secondary antibody. The specificity of silencing will be analyzed by measuring EGFR *actin* levels by western analysis following transfection of the dsRNA for *c-met*. The stress response will also be analyzed following silencing of *c-met* to ensure the continued lack of induction *in the cell line containing the Flp-In cloning site.*

Develop functional assay for cell migration and invasion

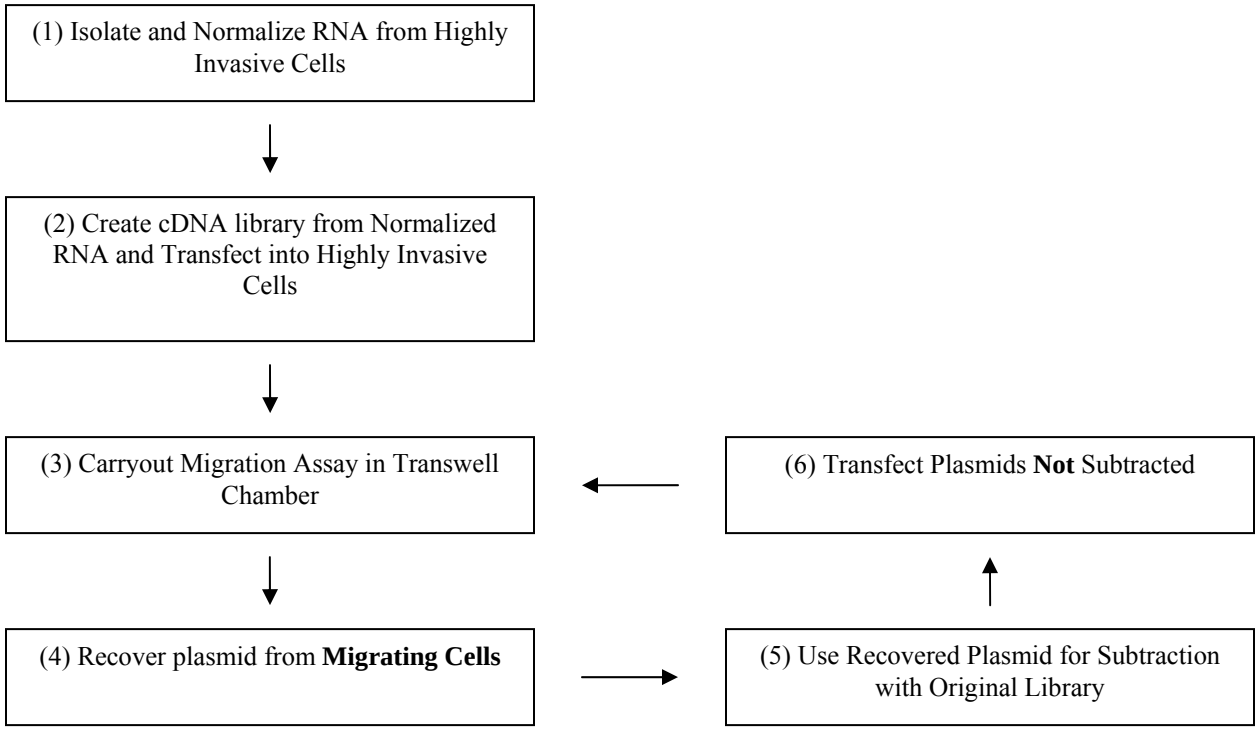
Prior to setting up the functional screen, it will be necessary to define conditions for optimal cell growth and migration. MDA-MB-231 cells will be plated in serum-free media in a 24-well Transwell culture chambers (Costar) with type IV collagen coated membranes[xcvii]. The lower chamber will contain 20% fetal bovine serum as a chemoattractant. Non-invasive (NIH3T3) and poorly invasive (MCF7) cells lines will be analyzed in parallel as negative controls for invasiveness. Initially, triplicate cultures of half-log order dilutions from 10^2 to 10^6 cells per well will be plated. Cells will be recovered by scrubbing with a sterile cotton swab in fresh culture media and seeded in 96-well plates. The number of invasive cells will be quantified using the Cell Titer 96 Aqueous One Cell Proliferation Assay (Promega). The above assay is a measure of migration. To measure true invasiveness, matrigel (BD) will be used.

Once the appropriate cell densities for the assay have been determined, cells (MDA-MB-231 and NIH3T3) will be mixed at different ratios to determine the sensitivity of identifying non-invasive cells. MDA-MB-231 cells will be treated with doxorubicin as a positive control for inhibition of invasiveness. In addition, MDA-MB-231 *containing the Flp-In integration site and stably transfected with either c-met or luciferase will be analyzed as described in Section C above for segregation of the plasmid to either the migrating or non-migrating population. Assuming there are ~100,000 expressed sequences in an MD-MBA-231 and 10 of these are involved in invasion, the luciferase silenced cells will be mixed at a 10,000:1 ration with the c-met silenced cells and the proportion in the migrating and non-migrating populations will be analyzed. Ideally, all the luciferase containing cells will be in the migrating population while the c-met silenced cells will lose the ability to migrate due to the knockdown of c-met and thus will remain in the top, non-migrating chamber. If, as*

anticipated, the non-migrating cells still contain a significant proportion (>50%) of luciferase knockdown cells, the cells from the upper chamber will be propagated and the assay repeated. This serial selection will continue until the non-migrating population contains at least 50% of cells containing the c-met dsRNA.

Negative Selection for Cell Migration

The issue of cells non-migrating even in a cell line that has been selected for high migration is real and the above experiments attempt to overcome this issue by continued rounds of selection. If this proves impossible to enrich the non-migrating population significantly, a second strategy based on negative selection and subtractive hybridization will be employed. The premise behind this strategy is that cells that continue to migrate across the membrane should contain only dsRNAs that ***are not*** *involved in migration. dsRNAs that silence important genes necessary for migration will segregate in the top chamber, along with cells that did not migrate for reasons other than silencing of important genes. The plasmids from the non-migrating cells will then be used to subtract plasmids from the original library. Those plasmids that are not subtracted out should represent genes involved in migration or lethality. This process will be repeated until the migrating population contains few (<50), if any, integrants. This strategy is far more complicated but is based on a number of standard methodologies. The flow chart below summarizes this strategy:*



The initial library will be created by normalize the RNA (1) by RoT[xcviii,xcix]. This normalization will decrease the complexity of the library and the number of unique clones that need to be analyzed. The normalized RNA will be used to generate cDNA and restriction digested, as described elsewhere in this proposal, to create the dsRNA library. The library will then be transfected into the Flp-In MDA-MB-231 cells (2) and the migration assay will be carried out (3). Unlike the functional assay described above, this strategy will involve isolation and recovery of plasmids from the migrating cells (4). DNA from the plasmid will be amplified by PCR using primers to the U6 and H1 promoters and the amplified DNA will be bound to a column and used to subtract[c] (5) the DNA from denatured plasmids generated in step (2). The DNA that is not subtracted, ie, the flow-through, will be amplified by PCR using U6 and H1 primers, a new library can be created and transfected into the MDA-MB-231 Flp-In cell lines (6) and the migration selection process (3) will be repeated. The plasmids recovered from the second round of selection will be used to subtract the subtracted library. This should ensure that remaining plasmids are involved in migration. For example, if the complexity of RNA from highly invasive MDA-MB-231 cells is equal to 5,000 unique mRNAs and there are 10 that are critical to migration, in the first migration assay, the cells that migrate through the membrane will contain 4,990 different plasmids. The cells that don't migrate will contain the 10 relevant plasmids and 99% irrelevant plasmids. Using the plasmids from the migrating cells to subtract material from the original library will, in an ideal situation, allow the 10 relevant genes to flow through (not be subtracted) and these will be used to create a second library. When this second library is transfected, no migrating cells should contain plasmids. In

practice, there are likely to be irrelevant plasmids that flow through thus this process will be repeated until fewer than 50 non-subtracted clones are found in the migrating population. Clones from this library will then be analyzed for their ability to inhibit migration.

Similar to the functional assay, c-met and luciferase will be used to validate this strategy. A normalized library will be created from the MDA-MB-231 cells that express luciferase and levels of luciferase, c-met and actin RNA will be monitored before and after normalization (1). Following selection, luciferase plasmid should be found in the migrating population whereas c-met should be found preferentially in the non-migrating population (3). While the original library should have contained a 1:1:1 ratio of luciferase:c-met:actin, the subtracted library should be enriched in c-met relative to luciferase or actin.

Aim 2. Generate dsRNA library from invasive MDA-MB-231 cell line

After the recombination sensitive cell line, expression and transfection methods and functional assay are developed, the dsRNA library will be generated and used to transfect the MDA-MB-231 cells. To ensure that genes involved in cell invasion are present, the most invasive cells will first be selected and used to obtain poly(A)+ RNA. The resulting cDNA will then be cloned into the appropriate vector and the library will be amplified for use in future transfections.

Select population of highly invasive cells from matrigel and isolate poly(A)+ mRNA

MDA-MB-231 cells will be plated in matrigel-coated Transwell plates at a cell number determined above and invasive cells will be isolated from the gel. The cells will be grown up in 96-well plates and individual wells will be replated in the matrigel-coated Transwell plates. Cells will be continually selected and replated in the matrigel-coated Transwell plates until a population of >99% invasive cells is obtained. This highly invasive clone will be propagated to 10^8 cells, a small number retested to ensure that they remain highly invasive and the rest used to isolate poly(A)+ RNA. The isolation of poly(A)+ RNA will be carried out using the Poly(A)Purist mRNA purification kit (Ambion) according to the manufacturer's instructions. The integrity of the RNA will be checked by analysis on a formaldehyde gel and northern analysis of *c-met*. RNA will be stored in 10-20 μl aliquots at -70^o until use.

Generate cDNA library using vector and poly(A)+ mRNA from above

The poly(A)+ RNA isolated from highly invasive MDA-MB-231 cells will be used to generate cDNA using the Universal RiboClone cDNA synthesis system (Promega). Briefly, first strand cDNA will be generated from 2 μg of poly(A)+ RNA using AMV reverse transcriptase. Second strand synthesis will be primed using random oligonucleotides and T4 DNA polymerase. A small amount of sample will be analyzed on an agarose gel for product size and by Southern using *c-met*. The high quality cDNA will then be digested with *Alu*I to generate ~250 base pair blunt end fragments, a fraction of which will be analyzed by gel electrophoresis and Southern analysis as above. Adaptors will then be ligated to these inserts to facilitate cloning into the Frt-comtaining plasmid generated above. *Eco*RI adaptors are provided with the RiboClone kit and will be used if the polylinker contains a unique *Eco*RI site, however, pcDNA5/Frt contains two sites and thus if this plasmid is used to subclone the convergent promoters, an alternate cloning site will be identified. Adaptors will be generated by synthesizing unphosphorylated oligonucleotides (Invitrogen) with the appropriate overhang to anneal to the alternate cloning site. After ligating these to the *Alu*I digested cDNA library, T4 polynucleotide kinase will be used with ATP to phosphorylate the inserts and the library will be cloned into the expression vector.

The library will be transformed into OmniMax bacterial cells (Invitrogen) and grown overnight on agar plates containing ampicillin. The number of individual colonies will be counted to determine the number of novel members in the library. To clone a transcript present at a level of 0.001% of the total mRNA present in the cell, 300,000 members would be required to produce 3x coverage of the poly(A)+ population. Since an average cDNA will be digested to six 250 base pair fragments by *Alu*I, 1.8 x 10^6 members will be needed to ensure coverage. Thus, the poly(A)+ purification, cDNA synthesis, cloning and transformation process will be repeated until the library reaches a complexity of 1.8 x 10^6 individual members. The colonies will then be scraped from the plates and the library amplified overnight in LB broth. EndoFree Plasmid Giga kits (Qiagen) will be used to purify the plasmids for determination of plasmid concentration and subsequent transfection into host cells prepared above.

Aim 3. Identify novel anti-invasive targets

The goal of this specific aim will be to identify the genes involved in cell migration and/or invasion. Following the generation of an RNAi expression library, the library will be transfected into the stable Flp-In MDA-MB-231 cells and at the appropriate time post-transfection, cell migration will be analyzed. Cells which are no longer capable of migrating across the membrane will be isolated and the identity of the dsRNA will be determined by sequencing.

Transfect library from Aim 2 into stable Flp-In cell lines developed in Aim 1 and select for stable recombinants

A population of highly invasive MDA-MB-231 host cells developed above will be co-transfected with the RNAi cDNA library (pRNAi) and the pOG44 vector (the vector encoding the recombinase (Invitrogen)). Prior to transfection, this population of cells will be analyzed for hygromycin B sensitivity. Cells will then be transfected by a method determined above with pOG44 and the pcDNA5/Frt vector at 3:1, 6:1, 9:1, 12:1 and 15:1 ratios. The number of hygromycin B resistant colonies will be counted and the number of plasmid integrations for each population of cells transfected with a particular ratio will be determined. Based on the design of the Flp-In system, it is expected that resistance will only be obtained for those plasmids that integrate at the Flp site, but additional integrants are also possible, thus Southern analysis will be carried out to determine the number of integrants for each transfection ratio. The plasmid ratio resulting in the greatest number of single integrants will be used for transfection of pRNAi.

Following the co-transfection of pOG44 and pRNAi, cells will be maintained in growth media for 48 hours then split to 25% confluence and media containing hygromycin B at the minimal toxic concentration determined above will be added. Cells will be grown in the presence of hygromycin B until use in the cell invasion assay. Note, individual colonies will not be isolated in growing the cells, rather the population will be pooled.

Isolate non-invasive clones by cell invasion assay

Prior to transfection of the library, the transfection efficiency will be analyzed by co-transfecting GFP subcloned into pcDNA5/Frt with pOG44. The population will be analyzed 24, 48, 96, 120, 168 and 240 hours after transfection to determine the percentage of cells and the levels of expression in the population. It is predicted that at early times, there will be a large distribution of levels of expressed GFP within the population since non-integrated plasmids will be expressing GFP and that at later times a more homogenous population will be obtained consisting primarily of single integrants at the Flp site. The earliest time at which a homogenous population is detected will be used to carry out the invasion assay.

The invasion assay will be carried out essentially as described in Specific Aims 1 and 2, with the exception that cells will be isolated from the upper chamber. These cells represent those in the population which are no longer capable of migrating through the membrane. The cells will be plated at low density, individual colonies will be isolated and transferred to 96-well plates and the individual clones will be retested for their ability to migrate across the membrane.

Identify sequences from non-invasive clones

Genomic DNA from individual clones that are no longer capable of migrating across the membrane will be isolated using the DNeasy 96 tissue kit (Qiagen). Briefly, cells will be cultured in 96-well plates, lysed and transferred onto a DNeasy 96-well plate. The genomic DNA selectively binds to the silica-gel membrane while contaminants pass through. Following washing, the DNA is eluted in water or low-salt buffer and used for analysis of integrated RNAi sequences by PCR.

PCR will be carried out with both forward and reverse primers designed by selecting regions of the multi cloning site that flank the inserted DNA on an iCycler for 25 cycles. The presence of an insert will be confirmed on an agarose gel. Clones with inserts will be further analyzed by triple digesting the PCR product with *Hha*I, *Hpa*II, and *Sau3A*I at 37^{o}C in Multi-Core Buffer (Promega). The resulting fragments will be analyzed on a 12% polyacrylamide gel. Based on the restriction fragment patterns, clones representing each of the different restriction patterns will be sent off for sequencing using the same primers used in the PCR amplification. The sequences will then be compared against those in GeneBank for identification of the genes involved in cell migration using Vector NTI 9.

Test RNAi sequences in MDA-MB-231 for prevention of cell invasion

The identification of sequences from non-invasive MDA-MB-231 cells likely are responsible for the change in migration of the cell line, however, it is possible that these sequences integrated into an endogenous gene important for cell migration, knocking it out and leading to the change in the cells ability to migrate across a membrane. Thus, it will be important to confirm that the isolated sequences are actually responsible for inhibition of cell migration. Since the isolation of the sequences is predicated on their lacking deleterious effects on cell growth or viability, the dsRNA sequences are not likely to be toxic.

The amplified sequences identified above will be recloned into the same dsRNA expression vector and transfected individually into MDA-MB-231 cells. The ability to inhibit cell migration in the absence of inducing the stress response pathway will be monitored as described above. Once the particular eliRNA has been shown to have the desirable activity in the cell, additional sequences from the gene will be cloned into the expression vector and compared to the isolated sequence for the level of silencing of the target sequence as determined by northern analysis and western analysis when antibodies are commercially available and for their ability to inhibit migration and cell invasion with inducing the stress response pathway. All sequences will then be tested for their effects on gene expression using Clontech's toxicity array. Briefly, poly(A)+ will be isolated from transfected cells and used to prepare radioactively labeled cDNA. The labeled cDNA will then be used to probe the arrays and the pattern will be compared to that of non-transfected cells. While a Affymetrix chip can also be analyzed (a reader is available at the core facility at LSUHSC), it is likely that silencing of a particular gene will effect the expression of other genes and it is possible that the silencing of a single gene with two different fragments may have different effects on the transcription profile but it is unclear how these changes/differences can be used to select which region of a gene to pursue and thus, the toxicity array will be used to rank order the different regions based on their induction of genes associated with toxicity.

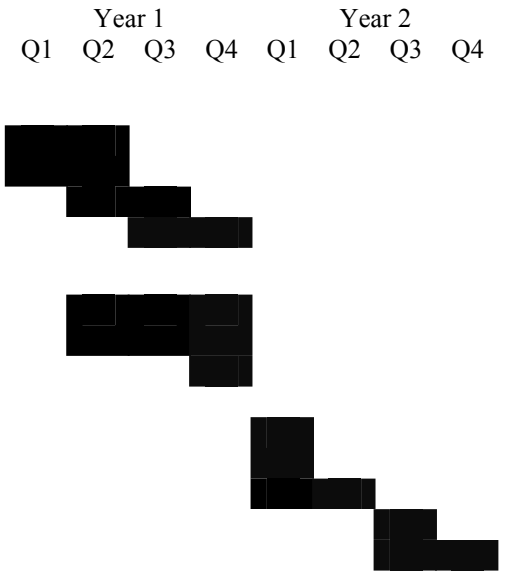
In addition to large dsRNAs, siRNAs against the particular target will also be analyzed. Pools of siRNAs are available for many human genes (Dharmacon) and will be purchased when available, along with the individual siRNAs, for each target identified above. For other genes in which pools are not available, at least three siRNAs will be designed using siDESIGN Center (Dharmacon). The pools and individual molecules will be transfected using Oligofectamine (Invitrogen) and effects on the target gene expression will be monitored using northern and western analysis at two and five days post-transfection. The siRNAs that result in >70% silencing of the target gene will be further tested for their ability to prevent cell migration without inducing the stress response pathway and for their effect on the induction of genes associated with toxicity.

The optimal RNAi molecules for each target, as defined by inhibition of target gene expression, ability to inhibit MDA-MB-231 cell migration, lack of induction of genes associated with toxicity and lack of induction of the stress response pathway, will be tested alone and in combination with RNAi molecules targeting other genes for effects on invasion as measured in the matrigel assay.

2. Milestones

The completion of this project will be carried out as follows:

Specific Aim:



Develop stable cell line, expression systems and functional assay for RNAi library evaluation

1.a. Create Flp-In plasmids for transfections, transfect and select stable MDA-MB-231 cells

1.b. Develop functional assay for cell invasion

1.c. Develop negative selection strategy

Generate dsRNA library from invasive MDA-MB-231 cell line

2.a. Select population of highly invasive cells from matrigel and isolate poly(A)+ mRNA

2.b. Generate cDNA library using vector and poly(A)+ mRNA from above

Identify novel anti-invasive targets

3.a. Transfect library into stable Flp-In cell lines developed & select for stable recombinants

3.b. Isolate non-invasive clones by cell invasion assay

3.c. Identify sequences from non-invasive clone

3.d. Test RNAi sequences in MDA-MB-231 for prevention of cell invasion

Specific milestones for each part of the project are as follows:

1.a. An established cell line containing an integrated Flip-in site will be propagated and analyzed. The clonal isolate expressing the highest level of β-gal *at least 10 fold over assay noise*, at a single integration site, and retaining the ability to migrate equal to the parental cell line, as determined by the % of cells capable of cell migration across the membrane, will be chosen for future cloning work.

1.b. The promoter system described will be analyzed first for their ability to silence a reporter gene and then for their ability to silence an endogenous gene. Maximal silencing of gene expression, at least to a level of 75% inhibition, will be used as a criteria for selecting the promoter. *This aim has been achieved and has been replaced with a new Aim 1c.*

1.b. Demonstration that a cell invasion assay can be established such that the percentage of MDA-MB-231 cells in a mixed population with NIH3T3 cells can be identified *and that non-migrating cells contain less than 50% of an irrelevant sequence (luciferase) when compared to a relevant sequence (c-met)..*

1c. *Demonstrate the negative selection strategy can enrich in plasmids that have been subtracted from plasmids obtained from migrating cells such that fewer than 50 irrelevant plasmids remain in the subtracted library.*

2.a. MDA-MB-231 isolates will be identified by continued selection that result in >99% migration across the membrane.

2.b. A cDNA library of 1.8 x 10^6 members will be generated from highly invasive MDA-MB-231 cellular mRNA.

3.a. Determine the number of single integrants and the % of cells transfected to identify the optimal ratio of library to recombinase plasmid to be used for transfections. Transfect the library and select the stable population with hygromycin B. Pool the population of cells such that the final population represents at least 3 million single integrants, as predicted by transfection efficiency.

3.b. Obtain at least 100 individual clones which can no longer migrate across the membrane by isolating non-invasive cells and growing colonies.

3.c. Use PCR to amplify sequences, obtain sequence information on at least 20 clones.

3.d. Using purified sequence to generate dsRNA, show that >70% of the target gene expression is inhibited and that the dsRNA is capable of inhibiting cell invasion by at least 70%.

Expected Critical Milestones: The concept under which this project is being initiated is that by creating a library of dsRNA molecules derived from a highly invasive breast cancer cell line, novel anti-invasive targets can be both identified and validated with the long-term intent of using these as targets for therapeutic intervention, perhaps through the use of the same dsRNA molecules used in their identification..

Deliverables, Milestone 1:	A vector system which can be used to generate dsRNA libraries for evaluation of functional targets. *This milestone has been achieved.*
Deliverables, Milestone 2:	A dsRNA library from a breast cancer cell line that can be used to analyze other functionally important cancer phenotypes.
Deliverables, Milestone 3:	Novel, validated therapeutics targets for the prevention of breast cancer cell invasion.

The first deliverable, the creation of an expression system, should be fully validated by the 3rd quarter of the first year. By developing a plasmid based system for expression of long dsRNA without induction of the interferon stress response pathway, a tool will be made available to efficiently silence gene expression. The use of long dsRNAs have been demonstrated to be more efficient at silencing gene expression in other systems and in preliminary experiments and the continued expression via convergent promoters will allow for long term analysis of gene function. The keys to successful completion of this milestone will be demonstrating effective silencing, lack of off-target effects and lack of induction of the stress response pathway.

The second important technology being developed in this project is the actual dsRNA library, which will be generated from cDNA made from the cell of interest, in this case, a highly invasive breast cancer cell. To completely validate this approach, sequences will need to be isolated and used to prove that they in fact led to the phenotypic change under study. Thus, the large dsRNA library validation will be completed during the 2nd half of year 2. Once fully validated, the use of such libraries will have broad applications, not only in cancer but for target identification and validation in a number of other diseases. For example, the receptor to a toxic ligand could be isolated by creating a dsRNA library from the cells expressing the receptor and transfecting these same cells with the dsRNA library, treating the transfected cells with the toxin and then isolating those cells which survive. This would be a tremendous advantage over transfecting a cDNA expression library from sensitive cells into resistant cells and looking for susceptibility to the ligand, i.e. cell death.

The final milestone, and perhaps most important from a therapeutic standpoint, will be the full validation of the target. This will involve optimizing the dsRNA, including analyzing actions of siRNAs derived from the same target, and carefully monitoring the stress response pathway and the induction of other genes associated with apoptosis and cell toxicity. The completion of the task by the end of year 2 will provide reagents necessary for initiation of *drug discovery* and preclinical development, with the long-term goal of developing a new class of anti-cancer drugs.

E. Human Subjects

Not Applicable.

F. Vertebrate Animals

Not Applicable

G. Literature Cited

[i] Reuters Business Insight (2003) The cancer market outlook to 2008. *Datamonitor PLC*.

[ii] Van de Vijver, M.J., He, Y.D., van't Veer, L.M., Dai, H., et al. (2002) A gene-expression signature as a predictor of survival in breast cancer. *The New England Journal of Medicine* 347:1999-2009.

[iii] Rowinsky, E.K. (2003) Signal events: cell signal transduction and its inhibition in cancer. *The Oncologist* 8:5-17.

[iv] Welch, D.R., Steeg, P.S., Rinker-Schaeffer, C.W. (2000) Review: Molecular biology of breast cancer metastasis Genetic regulation of human breast carcinoma metastasis. *Breast Cancer Research* 2:408-416.

[v] Sledge, G.W., Miller, K.D. (2003) Exploiting the hallmarks of cancer: the future conquest of breast cancer. *European Journal of Cancer* 39:1668-1675.

[vi] Hanahan, D., Weinberg, R. (2000) The hallmarks of cancer. *Cell* 100:57-70.

[vii] Gordon, L.A., Mulligan, K.T., Maxwell-Jones, H., Adams, M., Walker, R.A., Jones, J.L. (2003) Breast cell invasion potential relates to the myoepithelial phenotype. *International Journal of Cancer* 106:8-16.

[viii] Schafer, B., Gshwind, A., Ullrich, A. (2003) Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* on-line advance publication, December 1, 2003.

[ix] Yang, J.-M., Xu, Z., Wu, H., Zhu, H., Wu, X., Hait, W. (2003) Overexpression of extracellular matrix metalloproteinase inducer in multidrug resistant cancer cells. *Molecular Cancer Research* 1:420-427.

[x] Highleyman, L. (1998) FDA approves fomivirsen, famciclovir and Thalidomide. Food and Drug Administration. *BETA* Oct.5.

[xi] Matthews, S.J., McCoy, C. (2003) Thalidomide: a review of approved and investigational uses. *Clinical Therapeutics* 25:342-395.
[xii] Chen, W.-S., Liu, J.-H., Wei, S.-J., Liu, J.M., Hong, C.-Y., Yang, W.K. (2003) Colon cancer cells with high invasive potential are susceptible to induction of apoptosis by a selective COX-2 inhibitor. *Cancer Science* 94:253-258.
[xiii] Tsujii, M., Kawano, S., DuBois, R.N. (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastic potential. *Proceedings of the National Academy of Sciences, USA* 94:3336-3340.
[xiv] Shridhar, R., Zhang, J., Song, J., Booth, B.A., Kevil, C.G., Sotiropoulou, G., Sloane, B.F., Keppler, D. (2003) Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells. *Oncogene* Advanced online publication, December 15, 2003.
[xv] Coussens, L.M., Fingleton, B., Matrisian, L.M. (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295:2387-2392.
[xvi] Hjortoe, G.M., Peterson, L.C., Albrektsen, T., Sorensen, B.B., Norby, P.L., Mandal, S.K., Pendurthi, U.R., Mohan Rao, L.J. (2004) Tissue factor-factor VIIa specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated via PAR-2 and results in increased cell migration. *Blood* on-line advanced publication, January 8, 2004.
[xvii] Van Golen, K.L. (2003) Relationship between growth factor signaling and motility in aggressive cancers. *Breast Cancer Research* 5:174-179.
[xviii] Jenkinson, S.R., Barraclough, R., West, C.R., Rudland, P.S. (2004) S100A4 regulates cell motility and invasion in an *in vitro* model for breast cancer metastasis. *British Journal of Cancer* 90:253-262.
[xix] Holbro, T., Civenni, G., Hynes, N.E. (2003) The ErbB receptors and their role in cancer progression. *Experimental Cell Research* 284:99-110.
[xx] Ma, C., Lin, H., Leonard, S.S., Shi, X., Ye, J., Luo, J. (2003) Overexpression of ErbB2 enhances thanol-stimulated intracellular signaling and invasion of human mammary epithelial and breast cancer cells *in vitro*. *Oncogene* 22:5281-5290.
[xxi] Kim, E., Gunther, W., Yoshizato, K., Meissner, H., Zapf, S., Nusing, R.M., Yamamoto, H., van Meir, E.G., Deppert, W., Giese, A. (2003) Tumor suppressor p53 inhibits transcriptional activation of invasion gene thromboxane synthetase mediated by the proto-oncogene factor ets-1. *Oncogene* 22:7716-7727.
[xxii] Chau, N.-M., Ashcroft, M. (2004) Akt2: a role in breast cancer metastasis. *Breast Cancer Research* 6:55-57.
[xxiii] Wang, W., Wyckoff, J.B., Frohlich, V.C., Oleynikov, Y., Huttelmaier, S., Zavadil, J., Cermak, L., Bottinger, E.P., Singer, R.H., White, J.G., Segall, J.E., Condeelis, J.S. (2002) Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Research* 62: 6278-6288.
[xxiv] Jechlinger, M., Grunert, S., Tamir, I.H., Janda, E., Ludemann, S., Waerner, T., Seither, P., Weith, A., Beug, H., Kraut, N. (2003) Expression profiling of epithelial plasticity in tumor progression. *Oncogene* 22:7155-7169.
[xxv] Man, Y., Tai, L., Barner, R., Vang, R., Saenger, J.S., Shekitka, K.M., Bratthauer, G.L., Wheeler, D.T., Liang, C.Y., Vinh, T.N., Strauss, B.L. (2003) Cell clusters overlying focally disrupted mammary myoepithelial cell layers and adjacent cells within the same duct display different immunohistochemical and genetic features: implications for tumor progression and invasion. *Breast Cancer Research* 5:R231-R241.
[xxvi] Yoon, W.H., Song, I.-S., Lee, B.H., Jung, Y.-J., Kim, T.-D., Li, G., Lee, T.-G., Park, H.-D., Lim, K., Hwang, B.-D. (2004) Differential regulation of vimentin mRNA by 12-*O*-tetradecanoylphorbol 13-acetate and all-trans-retinoic acid correlates with motility of Hep 3B human heptocellular carcinoma cells. *Cancer Letters* 203:99-105.
[xxvii] Helbig, G., Christopherson, K.W., Bhat-Nakshatri, P., Kumar, S., Kishimoto, H., Miller, K.D., Broxmeyer, H.E., Nakshatri, H. (2003) NF-κB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *The Journal of Biological Chemistry* 278:21631-21638.
[xxviii] Camacho, L.H., (2003) Clinical applications of retinoids in cancer medicine. *J. Biol. Regul. Homeost. Agents* 17:98-114.; Orlowski, R.Z., Baldwin, A.S., Jr. NF-kappaB as a therapeutic target in cancer. *Trends in Molecular Medicine* 8:385-389.
[xxix] Kempen, I., Papapostololou, D., Thierry, N., Pochet, L., Counerotte, S., Masereel, B., Foidart, J.-M., Reboud-Ravux, M., Noel, A., Pirotte, B. (2003) 3-bromophenyl 6-acetoxymethyl-2-oxo-2*H*-Ibenzopyran-3-carboxylate inhibits cancer cell invasion *in vitro* and tumour growth *in vivo*. *British Journal of Cancer* 88:1111-1118.
[xxx] Fong, S., Ithahana, Y., Sumida, T., Singh, J., Coppe, J.-P., Liu, Y., Richards, P.C., Bennington, J.L., Lee, N.M., Debs, R.J., Desprez, P.-Y. (2003) *Id-1* as a molecular target in therapy for breast cancer cell invasion and metastasis. *Proceedings of the National Academy of Sciences, USA* 100:13543-13548.
[xxxi] Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K., Testa, J.R. (1996) Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2*expression and tumorigenicity by antisense RNA. *Proceedings of the National Academy of Sciences, USA* 93:3636-3641.
[xxxii] Grzmil, M., Hemmerlein, B., Thelen, P., Schweyer, S., Burfeind, P. (2003) Blockade of the cell type I IGF receptor expression in human prostate cancer cells inhibits proliferation and invasion, up-regulates IGF binding protein-3, and suppresses MMP-2 expression. *Journal of Pathology* 202:50-59.
[xxxiii] Yang, Y.-J., Zhang, Y.-L., Li, X., Dan, H.-L., Lai, Z.-S., Wang, J.-D., Wang, Q.-Y., Cui, H.-H., Sun, Y., Wang, Y.-D. (2003) Contribution of eIF-4E inhibition to the expression and activity of heparanase in human colon adenocarcinoma cell line: LS-174T. *World Journal of Gastroenterology* 9:1707-1712.
[xxxiv] Wu, S.-L., Hancock, W. S., Goodrich, G.G., Kunitake, S.T. (2003) An approach to the proteomic analysis of a breast cancer cell line (SKBR-3). *Proteomics* 3:1037-1046.
[xxxv] Celis, J.E., Gromov, P., Gromova, I., Moreira, J.M.A. et al. (2003) Integrating proteomic and functional genomic technologies in discovery-driven translational breast cancer research. *Molecular and Cellular Proteomics* 2:369-377.

[xxxvi] Miki, T., Fleming, T.P., Crescenzi, M., Molloy, C.J., Blam, S.B., Reynolds, S.H., Aaronson, S.A. (1991) Development of a highly efficient expression cDNA cloning system: application to oncogenes isolation. *Proceedings of the National Academy of Sciences, USA* 88:5167-5171.
[xxxvii] Chan, A.M., Fleming, T.P., McGovern, E.S., Chedid, M., Miki, T., Aaronson, S.A. (1993) Expression cDNA cloning of a transforming gene encoding the wild-type G alpha 12 gene product. *Molecular and Cellular Biology* 13:762-768.
[xxxviii] Leone, A., Flatow, U., VanHoutte, K., Steeg, P.S. (1993) Transfection of human nm23-H1 into the human MDA-MB-435 breast carcinoma cell line: effects on the tumor metastatic potential, colonization and enzymatic activity. *Oncogene* 8:2325-2333.
[xxxix] Nakanishi, K., Yong-Il, H., Ishiwatari, H., Takami, Y., Hayasaka, N., Yutsudo, M., Nojima, H., Hakura, A. (1993) Isolation of flat revertants from human papillomavirus type 18 E6E7 transformed 3Y1 cells by transfection with a rat embryo fibroblast cDNA expression library. *Cell Structure and Function* 18:457-465.
[xl] Feldman, R.J., Sementchenko, V.I., Gayed, M., Fraig, M.M., Watson, D.K. (2003) Pdef expression in human breast cancer is correlated with invasive potential and altered gene expression. *Cancer Research* 63:4626-4631.
[xli] Kissel, J.L., Kimichi, A. (1998) Death-associated proteins: from gene identification to the analysis of their apoptotic and tumour supporessive functions. *Molecular Medicine Today* 4:268-274.
[xlii] Dias, N., Stein, C.A. (2002) Antisense oligonucleotides:basic concepts and mechanisms. *Molecular Cancer Therapeutics* 1:347-355.
[xliii] Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., Baker, B.F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. *Journal of Biological Chemistry* 278:7108-7118.
[xliv] Stephens, A.C., Rivers, R.P. (2003) Antisense oligonucleotide therapy in cancer. *Current Opinion in Molecular Therapeutics* 5:118-122.
[xlv] Ho, P.T., Parkinson, D.R. (1997) Antisense oligonucleotides as therapeutics for malignant diseases. *Seminars in Oncology* 24:187-202.
[xlvi] Tolcher, A.W., Reyno, L., Venner, P.M., Ernst, S.D., Moore, M., Geary, R.S., Chi, K., Hall, S., Walsh, W., Dorr, A., Eisenhauer, E. (2002) A randomized Phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clinical Cancer Research* 8:2530-2535.
[xlvii] Banjerjee, D. (2001) Genasense (Genta Inc.) *Current Opinions on Investigative Drugs* 2:574-580.
[xlviii] Nahta, R., Esteva, F.J. (2003) Bcl-2 antisense oligonucleotides: a potential novel strategy for the treatment of breast cancer. *Seminars in Oncology* 30:143-149.
[xlix] Welch, P.J., Marcusson, E.G., Li, Q.X., Beger, C., Kruger, M., Zhou, C., Leavitt, M., Wong-Staal, F., Barber, J.R. (2000) Identification and validation of a new gene involved in anchorage-independent cell growth control using a library of randomized hairpin ribozymes. *Genomics* 66:274-283.
[l] Li, Q.X., Robbins, J.M., Welch, P.J., Wong-Staal, F., Barber, J.R. (2000) A novel functional genomics approach identifies mTERT as a suppressor of fibroblast transformation. *Nucleic Acids Research* 28:2605-2612.
[li] Suyama, E., Kawasaki, H., Nakajimi, M., Taira, K. (2003) Identification of genes involved in cell invasion by using a library of hybrid ribozymes. *Proceedings of the National Academy of Sciences, USA* 100:5616-5621.
[lii] Kashani-Sabet, M., Liu, Y., Fong, S., Desprez, P.-Y., Liu, S., Tu, G., Nosrati, M., Handumrongkul, C., Liggitt, D., Thor, A.D., Debs, R.J. (2002) Identification of gene function and functional pathways by systemic plasmid-based ribozyme targeting in adult mice. *Proceedings of the National Academy of Sciences, USA* 99:3878-3883.
[liii] Winston, W.M., Molodowitch, C., Hunter, C.P. (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295: 2456–2459.
[liv] Zamore, P.D., Tuschl, T., Sharp, P.A., Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101: 25–33.
[lv] Elbashir, S.M., Harbourth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.
[lvi] Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., Hannon, G.J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146-1150.
[lvii] Chopra, M., Pachuk, C., Satishchandran, C., Giordano, T. (2002) Using RNA interference to modulate gene expression. *Targets* 1:102-108.
[lviii] Shuey, D.J., McCallus, D.E., Giordano, T. (2002) RNAi: gene silencing in therapeutic intervention. *Drug Discovery Today* 7:1040-1046.
[lix] Shinomiya, N., Gao, C.F., Xie,Q., Gustafson, M., Waters, D.J., Zhang, Y.W., Vande Voude, G.F. (2004) RNA interference reveals that ligand-independent met activity is required for tumor cell signaling and survival. *Cancer Research* 64:7962-7970.
[lx] Kim, S.J., Johnson, M., Koterba, K., Herynk, M.H., Uehara, H., Gallick, G.E. (2003) Reduced c-Met expression by an adenovirus expressing a c-Met ribozyme inhibits tumorgenic growth and lymph node metastases of PC3-LN4 prostate tumor cells in orthotopic nude mouse model. *Clin. Cancer Res.* 9:5161-5170.
[lxi] Davies, G., Watkins, G., Mason, M.D., Jiang, W.G. (2004) Targeting the HGF/SF receptor c-met using a hammerhead ribozyme transgene reduces in vitro invasion and migration in prostate cancer cells. *Prostate* 60:317-324.
[lxii] Wang, X., Le, P., Liang, C., Chan, J., Keiwlich, D., Miller, T., Harris, D., Sun, L., Rice, A., Vasile, S., Blake, R.A., Howlett, A.R., Patel, N., McMahon, G., Lipson, K.E. (2003) Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. *Mol. Cancer Ther.* 2:1085-1092.
[lxiii] Christensen, J.G., Burrows, J., Salgia, R. (2005) c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. *Cancer Letters* 225:1-26.

[lxiv] Corso, S., Comoglio, P.M., Giordano, S. (2005) Cancer therapy: can the challenge be MET? *Trends Mol. Med.* 11:284-292.
[lxv] Clemens, M.J., Elia, A. (1997) The double-stranded RNA dependent protein kinase PKR: structure and function. *Journal of Interferon and Cytokine Research* 17:503-524.
[lxvi] Shirane, D., Sugao, K., Namiki, S., Tanabe, M., Iino, M., Hirose, K. Enzymatic production of RNAi libraries from cDNA. *Nature Genetics* 36:190-196.
[lxvii] Kiger, A.A., Baum, B., Jones, S., Jones, M.R., Coulson, A., Echeverri, C., Perrimon, N. (2003) A functional genomic analysis of cell morphology using RNA interference. *Journal of Biology* 2:27-27.15.
[lxviii] Caplen, N.J., Mousses, S. (2003) Short interfering RNA (SIRNA)-mediated RNA interference (RNAi) in human cells. *Annals of the New York Academy of Sciences* 1002:56-62.
[lxix] Sen, G., Wehrman, T.S., Myers, J.W., Blau, H.B. (2004) Restriction enzyme-generated siRNA (REGS) vectors and libraries. *Nature Genetics* 36:183-189.
[lxx] Berns, K., Hijmans, E. M., Mullenders, J., Brummelkamp, T. R., Velds, A., Heimerikx, M., Kekhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P.S., Beijersbergen, R.L., Bernards, R., (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature, 428:431-437.
[lxxi] Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S., Shultz, P. (2004) An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. PNAS 101:135-140.
[lxxii] Persengiev, S.P., Zhu, X., Green, M.R. (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 10:12-18.
[lxxiii] Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H., Williams, B.R.G. (2003) Activation of the interferon system by short-interefering RNAs. Nature Cell Biology 5:834-838.
[lxxiv] Mataraza, J.M., Briggs, M.W., Li, Z., Entwistle, A., Ridley, A.J., Sacks, D.B. (2003) IQGAP1 promotes cell motility and invasion. *Journal of Biological Chemistry* 278:41237-41245.
[lxxv] Czauderna, F., Fechtner, M., Aygun, H., Arnold, W., Klippel, A., Giese, K., Kaufmann, J. (2003) Functional studies of the PI(3)-kinase signaling pathway employing synthetic and expressed siRNA. *Nucleic Acids Research* 31:670-682.
[lxxvi] Chen, Y., Stamatoyannapoulos, G., Song, C.-Z. (2003) Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion *in vitro*. *Cancer Research* 63:4801-4804.
[lxxvii] Milhavet, O., Gary, D.S., Mattson, M.P. (2003) RNA interference in biology and medicine. *Pharmacological Reviews* 55:629-648.
[lxxviii] Giordano, T., Pachuk, C., Satishchandran, C. (2002) Use of post-transcriptional gene silencing for identifying nucleic acid sequences that modulate the function of a cell. *European Patent Application* EP 1 229 134 A2.
[lxxix] Paddison, P.J., Caudy, A.A., Hannon, G.J. (2002) Stable suppression of gene expression by RNAi in mammalian cells. *Proceedings of the National Academy of Sciences, USA* 99:1443-1448.
[lxxx] Billy, E., Brondani, V., Zhang, H., Muller, U., Filipowicz, W. (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proceedings of the National Academy of Sciences, USA* 98:14428-14433.
[lxxxi] Giordano, T., Foster, D.N. (1989) Identification of a Highly Abundant cDNA from Late Passage WI-38 Cells. *Experimental Cell Research* 185:399-406.
[lxxxii] Chandrasekaran, K., Stoll, J., Giordano, T., Atack, J.R., Matocha, M.F., Brady, D.R., Rapoport, S.I. (1992) Differential Expression of Cytochrome Oxidase (COX) Genes in Different Regions of Monkey Brain. *Journal of Neuroscience Research* 32:415-423
[lxxxiii] Foster, D.N., Gailhouse, D., Giordano, T., Min, B.-H., Lamb, I.C., Porter, D.A., Intehar, K.J., Bacon, W.L. (1992) Nucleotide Sequence of the cDNA Encoding the Common alpha Subunit of Chicken Pituitary Glycoprotein Hormones. *Journal of Molecular Endocrinology* 8, 21-27.
[lxxxiv] Doucette-Stamm, L., Monteggia, L.M., Donnelly-Roberts, D., Wang, M.T., Lee, J., Tian, J., Giordano, T. (1993) Cloning and Sequence of the Human α7 Nicotinic Acetycholine Receptor. *Drug Development Research* 30, 252-256.
[lxxxv] Monteggia, L.M., Gopalakrishnan, M., Touma, E., Idler, K., Nash, N., Arneric, S.P., Sullivan, J.P., Giordano, T. (1995) The Cloning and Transient Expression of the Human α4 and ß2 Neuronal Nicotinic Acetycholine Receptor (nAChR) Subunits. *Gene* 155:189-193.
[lxxxvi] Lin, Y.J., Carver, J.M., and Giordano, T. (1996) The Cloning and Expression in the CNS and Periphery of the Human NR2C NMDA Glutamate Receptor. *Molecular Brain Research* 43:57-64.
[lxxxvii] Elbashir, S.M., Lendeckel, W., Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes and Development* 15:188-200.
[lxxxviii] Ngo, H., Tschudi C, Gull K, Ullu E. (1998) Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. *Proceedings of the National Academy of Sciences, USA* 95: 14687-14692.
[lxxxix] Hartmann, R., et al., (1998) Activation of 2'-5' oligoadenylate synthetase by single-stranded and double-stranded RNA aptamers. *Journal of Biological Chemistry* 273:3236-3246.
[xc] Hunter, T., Hunt, T., Jackson, R.J., Robertson, H.D. (1975) The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *Journal of Biological Chemistry* 250:409-417.
[xci] Kostura, M., Mathews, M.B. (1989) Purification and activation of the double stranded RNA-dependent eIF-2 kinase DAI. *Molecular and Cellular Biology* 9:1576-1586.
[xcii] Kasim, V., Miyagishi, M., Taira, K. (2004) Control of siRNA expression using the Cre-loxP recombination system. Nucleic Acids Research 32:e66.

[xciii] *Kaykas, A., Moon, R.T. (2004) A plasmid-based system for expressing small interfering RNA libraries in mammalian cells. BMC Cell Biology 5:16.*
[xciv] *Kobayashi, N., Matsui, Y., Kawasw, A., Hirata, K., Miyagishi, M., Taira, K., Nishikawa, M., Takakura, Y. (2004) Vector-based in vivo RNA interference: Dose- and time- dependent suppression of transgene expression. JPET 308:688-693.*
[xcv] Tran, N., Cairns, M.J., Dawes, I.W., Arbdt, G.M. (2003) Expressing functional siRNAs in mammalian cells using convergent transcription. *BMC Biotechnology* 3:21 p1-9.
[xcvi] Hovnanian, A., Rebouillat, D., Mattei, M.G., Levy, E.R., Marie, I., Monaco, A.P., Hovanessian, A.G. (1998) The human 2',5'-oligoadenylate synthetase locus is composed of three distinct genes clustered on chromosome 12q24.2 encoding the 100-, 69-, and 40-kDa forms. *Genomics* 52:267-277.
[xcvii] Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., McEwan, R.N. (1987) Rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Research* 47:3239-3245.
[xcviii] Karnaoukhova,L., Tamg, P.-Z., Wang, D., Peng, L., Neiditch, B. (2004) Construction of nano-quantity, full-length and normalized cDNA libraries. *Bioscience Technology* www.biosciencetechnology.com.
[xcix] Bonaldo, M.F., Soares, M.B., Lennon, G. (1996) Normalization and subtraction: two approaches to facilitate gene discovery. *Genome Research* 6:791-806.
[c] Diatchenko, L., Lau, Y.-F. C., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Gurskaya, N.S., Siebert, P.D. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue specific cell probes and libraries. *PNAS* 93: 6025-2030.

H. Consortium/Contractual Arrangements: Not Applicable

I. Letters of Support: Letter from Consultant/Advisor, James Cardelli

J. Product Development Plan: Not Applicable